THE ASSOCIATION OF rs7903146(C/T) AND rs 12255372(G/T) POLYMORPHISMS OF THE TCF7L2 GENE WITH TYPE 2 DIABETES MELLITUS IN CHENNAI SUBURBAN POPULATION

Dissertation submitted to

THE TAMILNADU DR. M.G.R. MEDICAL UNIVERSITY

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CHENNAI.

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CERTIFICATE

This is to certify that this dissertation entitled **"THE ASSOCIATION OF rs7903146(C/T) AND rs12255372(G/T) POLYMORPHISMS OF THE TCF7L2 GENE WITH TYPE 2 DIABETES MELLITUS IN CHENNAI SUBURBAN POPULATION"** is the bonafide original work done by

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DECLARATION

I solemnly declare that this dissertation entitled "**The Association of rs7903146(C/T) and rs12255372(G/T) Polymorphisms of the TCF7L2 gene with Type 2 Diabetes Mellitus in Chennai SubUrban Population"** is bonafide work done by me in the Department of Biochemistry, Govt. Kilpauk Medical College, Chennai, under the guidance and supervision of **Prof. Dr.V.MEERA, M.D.,** Associate professor of Biochemistry, Govt. Kilpauk Medical College, Chennai–600 010.

This dissertation is submitted to **THE TAMILNADU Dr. M.G.R. MEDICAL UNIVERSITY,** Chennai, in partial fulfillment of the university regulations for the award of degree of **M.D BIOCHEMISTRY (BRANCH - XIII)** examinations to be held in **APRIL – 2015**.

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CONTENTS

INTRODUCTION

Diabetes is truly the most challenging health problem in this 21st century.[1]Type 2 Diabetes Mellitus is rapidly emerging as a modern epidemic[2] both in high income and low income countries. It is stated as a world public health problem and the global crisis due to diabetes threatens the health as well as the economy of every nation. The good news is, once the risk factors are identified the development of Type 2 Diabetes can be either delayed or even prevented by healthy lifestyle pattern and required medications. The goal is to reduce the disease and its economic burden and improve the quality of life for all persons who have, or are at risk of, Diabetes Mellitus.

The Greek Apollonius of Memphi first used the term "diabetes" or "to pass through" in 230 BC[146]. The Indian physicians, Sushruta and Charaka were the first to identify Type 1 and Type 2 Diabetes as two separate conditions in 400-500 AD[146]. In the late 17th century, the Britain John Rolle added the term "mellitus" or "from honey" to separate the condition from Diabetes Insipidus[146].

Type 2 Diabetes is a multisystem disorder due to defect in glucose metabolism causes multiple metabolic abnormalities manifesting in varying degrees. The glucose metabolism is well controlled by multiple hormones and neurotransmitters in response to nutritional, emotional and environmental changes. Unger first described diabetes, as a “bi-hormonal” disease characterized by insulin deficiency and glucagon excess[3].

The multi-hormonal view of glucose homeostasis through the pancreatic and incretin hormones let to a new understanding of Diabetes Mellitus[3]. The glucoregulatory hormones included are insulin, glucagon, amylin, glucagon like peptide-1, glucose–dependent insulinotropic peptide, epinephrine, cortisol and growth hormones[3].

Traditionally the pathophysiology of T2DM was focused on beta cell dysfunction and insulin resistance in liver and skeletal muscle. Now after many researches in the past two decades, revealed a basic understanding about mechanism and dysfunctions in gastrointestinal tract, pancreatic alpha cells, adipose tissue, brain and kidney that produced more robust picture of Type 2 Diabetes [4].

Despite the best efforts of intensively managing diabetes with insulin and antihyperglycemic drugs, there were unpredictable glucose fluctuations, hypoglycemia and weight gain leading to frustration and risk. Universally, World Health Organization (WHO) and American Diabetes Association (ADA) criteria are still used for screening and diagnosis.

Recent research into the pathophysiology of Type 2 Diabetes has led to the introduction of new medications like inhibitors of the sodium-glucose cotransporter 2 and 11ß-hydroxysteroid dehydrogenase 1, insulin-releasing glucokinase activators and pancreatic-G-protein-coupled fatty-acid-receptor agonists, glucagon like peptide-1 analogues, dipeptidyl peptidase-IV inhibitors, metabolic inhibitors of hepatic glucose output, glucagon-receptor antagonists and quick-release bromocriptine [5].

Even after the invention of many new drugs, the prevalence of Type 2 Diabetes is still in the higher end. According to International Diabetic Federation 2014, nearly 183 million people are still unaware that they are living with diabetes[17]. Therefore, the identification of individuals at high risk of getting diabetes is of great importance for investigators and health care providers.

Type 2 Diabetes being a multi-factorial genetic syndrome and the advancing genotyping technology in the last 5years, have facilitated rapid progress in genetic studies in T2DM. Since 2007, more than 65 genetic variants have been identified by Genome-Wide Association Studies (GWAS). They were found to increase the risk of T2DM by 10–30%[6]. Most of these variants are non-coding variants, and identifying their functional consequences is a real challenge to investigators. Many of the variants identified are involved in the regulation of insulin secretion and not insulin action in insulin-sensitive tissues. However, how genetics involve in the development of diabetes is poorly understood.

The major issue to address in Diabetes biology is to identify the genetic changes in the disease and their occurrence in different populations. Variants of a number of genes have been associated to T2DM among Europeans. However, the contributions of these genetic variants in other ethnic groups are unclear. Reports from several Indian populations give a heterogeneous picture owing to its diverse ethnicity.

A large amount of data available on the genetics of T2DM from association studies of candidate gene variation include variants of :

1. Calpain-10 -**CAPN10**, Horikawa et al [7] .,
2. Peroxisome proliferator-activated receptor gamma -**PPARG**, Agostini et al [8]
3. Potassium inwardly rectifying channel, subfamily J, member 11-**KCNJ11,** Florez et al.,
4. ATP binding cassette, subfamilyC, member 8 - **ABCC8**, Van Dam RM et al .,
5. Hepatocyte nuclear factor-1A - **HNF1A**, Triggs et al.,
6. Hepatocyte nuclear factor-4A - **HNF4A**, Muller et al.,
7. Glucokinase –**GCK**, Weedon et al.,
8. Plasma cell glycoprotein1/encoding ectonucleotide pyrophosphate phosphodiesterase 1 -**PC-1**/**ENPPI**, Bottcher et al.,
9. Insulin receptor substrate-1 -**IRS-1**, Hitman et al.,
10. Protein tyrosine phosphatase 1B –**PTPNI**, Florez et al.,
11. The nuclear lamina gene-**LMNA**,Owen et al.,
12. Transcription factor 7-like 2 **-TCF7L2,** Grant et al.,
13. Insulin like growth factor 2 mRNA binding protein 2 -**IGF2BP2**, Zeggeni et al.,
14. KQT like subfamily, member 1 gene-**KCNQ1**,Tan et al., [9].

Among all the association studies with T2DM in various populations including Indian populations, **TCF7L2** has been shown to be most promising in South Indian ,Bodhini et al.,[10] and Western Indian, Chandak et al.,[11] populations, where intronic SNP (rs7903146, rs12255372, rs4506565) show association with T2DM.

TCF7L2 gene , a transcription factor is critical for expression of glucagon like peptide-1, an incretin hormone in intestinal endocrine cells that mediates insulin synthesis and secretion in β cells of pancreas [12], β cell proliferation, neogenesis and protection against apoptosis.

Hence it is proposed to study the association of TCF7L2 gene polymorphism with Type 2 Diabetes and also to correlate with GLP-1 level and insulin level .

AIM OF THE STUDY

Glucagon like peptide-1 has significant role in endocrine pancreatic hormone secretion through the enteroinsulinar axis. The transcription factor TCF7L2 is an important mediator for the Proglucagon gene transcription and GLP-1 production.

With these salient mechanisms in Insulin secretion and maintenance of glucose homeostasis, the aim of this study is to find an association:

1. Between TCF7L2 gene polymorphism at two restriction sites and Type 2 Diabetes Mellitus in cases and controls.
2. Between postprandial GLP-1 level in circulation and TCF7L2 gene polymorphism.
3. Between Insulin, HbA1c and Glucose level in blood and TCF7L2 gene polymorphism.
4. Between TCF7L2 gene – T allele and its associated risk of Type 2 Diabetes.
5. Between T allele and GLP-1 level in blood in cases and controls.
6. By logistic regression to assess the individual predictability of various risk factors for the development of Type 2 Diabetes Mellitus.
7. To study the distribution of risk T allele of TCF7L2 gene in the study population.

REVIEW OF LITERATURE

Diabetes is defined as a metabolic disorder due to heterogeneous etiology which presents with chronic hyperglycemia along with disturbances in all three macro molecules carbohydrate, protein, lipid metabolism where the major defect can be in insulin secretion, insulin action or both[13]. Degree of hyperglycemia is the main determinant for development of complications like diabetic retinopathy, diabetic nephropathy and diabetic neuropathy. In recent trend children, adolescents and young adults are becoming the target for Type 2 diabetes and pre diabetes, in many countries including Japan, USA, India, Australia and UK, Bloomgarden et al., .The decrease in the age of onset of Diabetes is of great concern, as the main burden is on future generations.

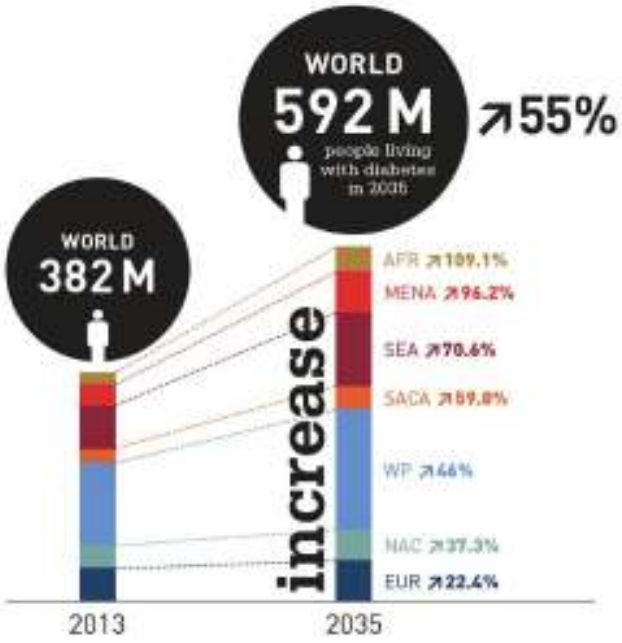
The cause for diabetes is enclosed in a complex group of genetic and epigenetic systems and its interaction with equitably complex environmental and behavioral factors [14]. Diabetes Mellitus is an ambulatory "care sensitive condition" [15]. The consequences of diabetes are, it reduces life expectancy by nearly 15 years, it increases the risk of CVD by 2 to 4 times, and for kidney failure, lower limb amputations, adult – onset blindness, Diabetes is the leading cause. In addition to these human cost, the costs of medical care, disability ,and premature death increase the financial cost due to type 2 diabetes.

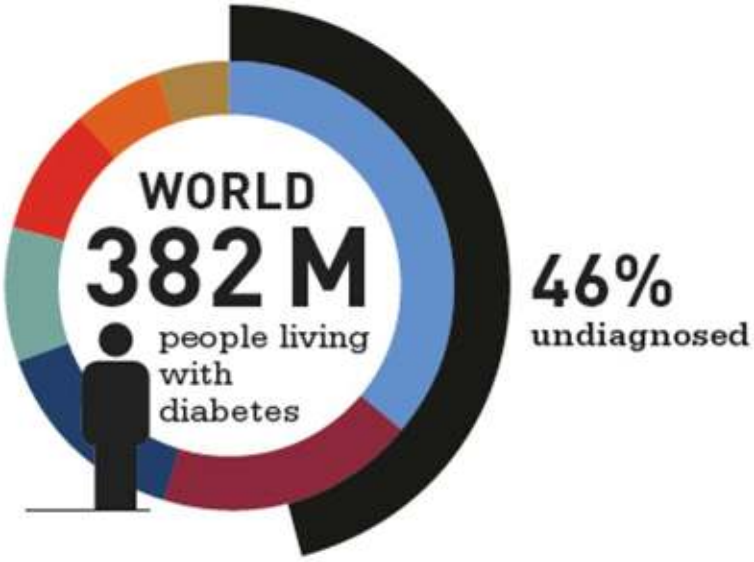
Epidemiology:

Diabetes mellitus is the most important health challenge to all nations that is increasing in an alarming rate all over the world [14]. The Prevalence of Type 2 Diabetes was around 171 million in 2000, which has almost doubled in past decades .According to the International Diabetes Federation(IDF) –sixth diabetic atlas[147], the World diabetic prevalence in 2013 is estimated to be 382 million and is expected to increase to 592 million by 2035 where the rate of increase is 55%. Nearly 175 million people are currently undiagnosed and unknowingly progressing towards complications. The estimated rate of increase in prevalence was 69% in developing countries and 20% in developed countries [17]. This shows the growing burden of diabetes in developing countries like India. According to IDF – number of individuals with Type 2 Diabetes in 2013 are - China – 98.4 million , India -65.1 million and United states – 24.4 million, showing the largest number of diabetic population in the world.

In India, Type 2 Diabetes is erupting like a Pandemic. IDF estimated that the total number of diabetic subjects to be around 50.8 million in 2010 and expected to rise to 87 million by 2030 [18]. In India, people both in rural and urban regions were affected by diabetes, but Urban India has a higher prevalence of 5.6% than Rural India with 2.7% [19]. The high prevalence observed in urban area shows that the rapid urbanization plays a major role. The most worrying trend in incidence of diabetes is its shift towards younger age where Diabetes commences ten years earlier than in western countries[20]. In Chennai, diabetic prevalence was 13.5% in 2000 that went up to 14.3% in 2004 and reached 18.6% in 2006 [20]. The primary cause for the epidemic of

INTERNATIONAL DIABETIC FEDERATION 2014





diabetes is the rapid transition of culture associated with dietary changes and physical inactivity which are evident from the increased diabetic prevalence in the urban population like Chennai.

Classification:

ADA gave the detailed classification for diabetes based on etiology that was published in the year 2014.

Diabetes Mellitus - Etiologically classified into four categories [(22)] :

I. Type 1 Diabetes (usually show absolute insulin deficiency due to β cell destruction).

A. Immune mediated.

B. Idiopathic.

II. Type 2 Diabetes(caused mainly by insulin resistance along relative insulin deficiency or by insulin secretory defect along with insulin resistance)

III. Other Types

A. Genetic and Epigenetic defects of β- cells

1. Chromosome 12, HNF-1a (MODY3)
2. Chromosome 7, glucokinase (MODY2)
3. Chromosome 20, HNF-4a (MODY1)
4. Chromosome 13, insulin promoter factor-1 (IPF-1; MODY4)
5. Chromosome 17, HNF-1b (MODY5)
6. Chromosome 2, NeuroD1 (MODY6)
7. Mitochondrial DNA
8. Others

B. Genetic defects in insulin action

C. Diseases of the exocrine pancreas

D. Endocrinopathies

E. Drug or chemical induced

F. Infections

G. Uncommon forms of immune-mediated diabetes

H. Other genetic syndromes sometimes associated with diabetes

IV. Gestational diabetes mellitus.

Disorders of Glycemia : Etiological category and stages:[21]

1. Normoglycemia:

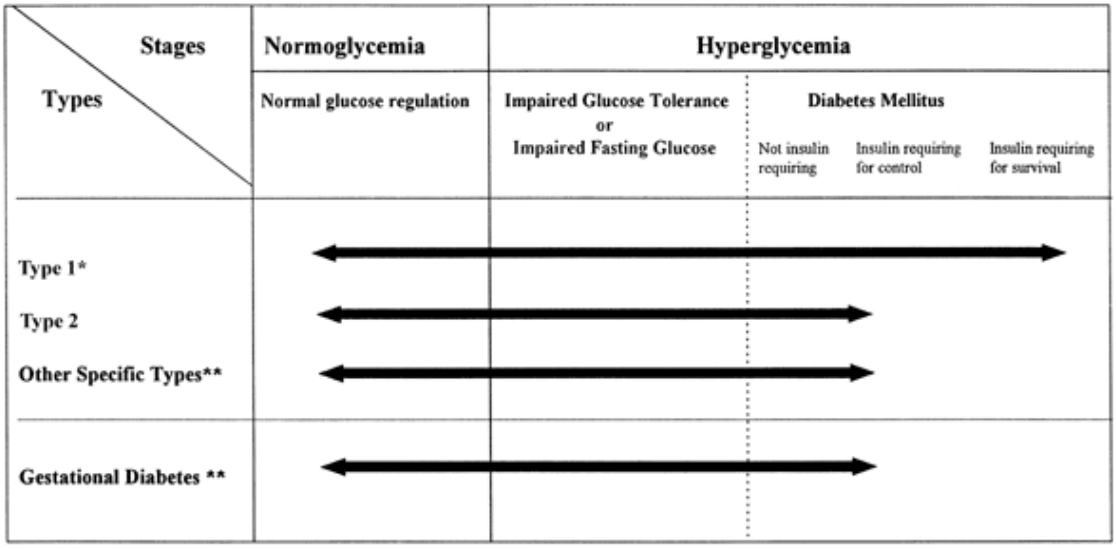
   Regulated normal blood glucose level.

2. Hyperglycemia in Prediabetic stage:

   Includes impaired glucose tolerance and impaired fasting glucose .

3. Hyperglycemia with Frank diabetic stage:

   High blood glucose level – unregulated.



Among the various types of diabetes, Type 2 Diabetes accounts for 90% to 95% of diabetic cases and Type 1 Diabetes accounts for 5%-10% only[21].

Type 1 Diabetes Mellitus:

Type 1 diabetes is immune mediated disorder ,also known as insulin dependent diabetes or juvenile onset diabetes. Prevalence of type 1 diabetes was found to be less than 1% in most population. But the worldwide increase in incidence of Type 1 Diabetes was at a rate of 3% per year (Onkamo et al., 1999)[21]. Holt, 2004 estimated that nearly 20 million people worldwide, were affected by type 1 diabetes.The incidence of Type 1 Diabetes was more among childhood and adolescent age groups, but was also found in late eighties and nineties[21].

There is destruction of beta cells of pancreas due to cell mediated autoimmune reaction. The auto antibodies are found against Islet cells, Insulin, Glutamic Acid Decarboxylase (GAD) and Trypsin IA-2 & IA2β . In 85 to 90 % of diabetic cases , one and mostly more of these autoantibodies are present. The proven genetic causes for Type 1 Diabetes are strongly HLA associated with linkage to DQA and DQB genes. A recent study by Dotman et al., showed a distinct seasonal variation in incidence of Type 1 Diabetes in various countries with higher rates in cold winter season and lower rates in warm summer months.[22]

Risk factors :

Among various environmental risk factors of Type 1 DM, changing global environment points out an important reason for recent temporal increase of type 1 DM incidence. Other environmental risk factors for type 1 DM that has received more attention recently are viruses and infant nutrition. Various viral infections that increase the subsequent risk of developing Type 1 Diabetes are Coxsackie virus B (Dahlquist et al., 1998), rotavirus (Honeyman et al., 2000), cytomegalovirus (Pak et al., 1988),mumps (Hyoty et al., 1993) and rubella.T1D children were found being breast fed for shorter period of time than non diabetic children (Borsh-Johnsen et al., 1984)[22]

Type 1 Diabetes, a chronic T-cell mediated autoimmune disease that causes destruction of pancreatic beta –cells leading to progressive and irreversible failure of insulin secretion. Type 1 diabetes mostly presents with diabetic ketoacidosis (DKA). Due to lack of insulin, rate of glycogenolysis and gluconeogenesis in the liver is increased leading to high glucose concentration. This causes osmotic diuresis with symptoms of polyuria, polydypsia and polyphagia. Lack of insulin causes increased lipolysis in adipose tissue and accumulation of ketone bodies progressing to diabetic ketoacidosis.

According to The American Diabetes Association's (ADA) 2014 [22] :

Plasma blood glucose and A1C goals for type 1 diabetes by age-group

Values by age (years)	Plasma blood glucose goal range(mg/dL)			Rationale
	Before meals	Bedtime/overnight	A1C	
Toddlers and preschoolers (0–6)	100–180	110–200 ,	<8.5%	-Vulnerabilityt hypoglycemia -Insulin sensitivity -Unpredictability in dietary intake and physical activity -A lower goal (<8.0%) is reasonable if it achieved without excessive hypoglycemia
School age (6–12)	90–180	100–180	<8%	-Vulnerability of hypoglycemia - A lower goal (<7.5%) is reasonable if it can be achieved without excessive hypoglycemia
Adolescents and	90–130	90–150	<7.5%	-A lower goal (<7.0%) is reasonable if it can be achieved young adults (13–19) without excessive hypoglycemia

Type 2 Diabetes Mellitus :

Type 2 Diabetes Mellitus also referred as non insulin dependent diabetes or adult onset diabetes that shows chiefly insulin resistance along with relative rather than absolute insulin deficiency. The specific etiology of this type of diabetes is not known. Initially with beta cell functions being normal, the insulin resistant patients show high blood glucose level with insulin level appearing normal, and later the insulin secretion becomes defective and inability to compensate for the insulin resistance. As majority of Type 2 Diabetics are obese, Obesity itself could be a cause for insulin resistance. Obesity due to increased abdominal fat predominantly contributes to insulin resistance than the traditional weight criteria, leading to Type 2 Diabetes. The presentation of Type 2 Diabetes is extremely critical and tricky. Most of the time T2DM remains undiagnosed for years with hyperglycemic blood status without any classical symptoms. It seldom presents with ketoacidosis unless there is stress of illness such as infections. But the inexorable risk of T2DM patients is development of micro and macro vascular complications [21].

Diagnostic Criteria :

ADA- Standards of Care stated the following diagnostic criteria for Type 2 Diabetes in 2014 :[22]

ADA - 2014 - Criteria for the diagnosis of diabetes

A1C $\geq$ 6.5%. The test should be performed in a laboratory using a method that is NGSP certified and standardized to the DCCT assay.*
OR
FPG $\geq$126 mg/dL (7.0 mmol/L). Fasting is defined as no caloric intake for at least 8 h.*
OR
Two-hour PG $\geq$200 mg/dL (11.1 mmol/L) during an OGTT. The test should be performed as described by the WHO, using a glucose load containing the equivalent of 75 g anhydrous glucose dissolved in water.*
OR
In a patient with classic symptoms of hyperglycemia or hyperglycemic crisis, a random plasma glucose $\geq$ 200 mg/dL (11.1 mmol/L).

An International expert committee on Diagnosis and Classification of Diabetes Mellitus did extensive review and included HbA1c in diagnostic criteria that must be measured using the method that is certified by National Glycohemoglobin Standardisation Program (NGSP) and results must be tracable to the Diabetic Control and Complications Trial (DCCT) reference assay.[22]

The Expert Committee on Diagnosis and Classification of Diabetes Mellitus gave the criteria for Increased risk for Diabetes category - **Pre Diabetics**[22]:

1. Fasting plasma glucose 100 – 125 mg/dl (5.6 – 6.9 mmol/L)

   Stated as Impaired Fasting glucose (IFG).

or

2. Two hours Plasma Glucose in the 75-gms OGTT

   140 – 199 mg/dl (7.8 – 11.0 mmol/L)

   Stated as Impaired Glucose Tolerance (IGT)

or

3. A1C 5.7-6.4 %

Risk factors :

The risk factors for Type 2 diabetes are classified as:

1. non modifiable (genetic) and
2. modifiable (environmental) risk factors

Non modifiable Risk factors:

1. Age:

The chances of developing diabetes was found to be extremely high after forty years of age.

2. Race, Ethnicity and Family history :

Many inherited genetic factors were identified that exposes certain ethnic groups with high chances of developing diabetes .(Asians, Africans, Alaskan natives, American Indians, Hispanic Americans, Pacific islanders descend).

Modifiable Risk factors:

1. Obesity:

To estimate body fat and its distribution Body Mass Index, Waist circumference and Waist hip ratio are measured which are the health risks for developing diabetes. Evidences show obesity as the major drive of Type 2 Diabetes [24].Ectopic fat deposits leads to hepatic insulin resistance which is found to precede Type 2 Diabetes manifestation[24].

2. Dyslipidemia:

Recent researches showed two to four times increased risk of getting CAD in diabetic subjects [23] .CAD was prevalent more in those diabetic patients with isolated high cholesterol, high LDL and low HDL [25] .

3. Hypertension:

In Hypertensive subjects the risk of diabetes is 2.5 times more than in normotensive subjects.

4. Dietary habits:

Dietary fibres is one of many factors that affect post prandial glucose and insulin receptor. An inverse relationship is found between blood insulin levels and dietary fibres [27]. Diet rich in non starch polysaccharides , ώ-3 fatty acids , low glycemic

index foods , micronutrients like vitamin E, chromium, magnesium decrease the risk of Type 2 Diabetes.[26]

Type 2 Diabetes has strong genetic reason. However the genetics of diabetes is complex and yet to be clearly defined. The cause for inability of pancreas to secrete enough insulin or insulin produced showing resistance would ultimately be genetic (non-modifiable risk factor) but not every person who has that particular genetic abnormality will develop Type 2 Diabetes as other risk factors along with life style choices (modifiable risk factors) have great influence on it. Even for those having strong family history or belonging to certain ethnic groups with inheritable risk factors for Type 2 Diabetes, the disorder may or may not manifest depending on their lifestyle pattern and environmental factors. By following healthy lifestyle pattern like increased physical activity, regular exercise and taking balanced diet have showed nearly 60% decrease in risk of progression of IGT to T2D in contrast to nearly 30% reduction in risk of progression to T2D by taking oral hypoglycemic drugs (Diabetes Prevention Program Study Group, 2002, US). Diet and physical activity play critical role in the management of (ABC) A1c, Blood pressure, Cholesterol in Type 2 Diabetic patients. Thus the metabolic pathway of macro and micro molecules ingested in diet has to be under strict regulation.

Pathophysiology behind Diabetes :

Diabetes is a metabolic disorder with chronic hyperglycemia and the major defect is found in the metabolic pathway of carbohydrate which constitutes the major part of physiological system. Carbohydrates are not only major energy providers but

also needed for specific cellular functions and protein modifications like glycosylation. Hence it is highly essential for the body to maintain the blood glucose levels within limits which is done by many hormones of which the most significant action is exerted by the hormone Insulin .

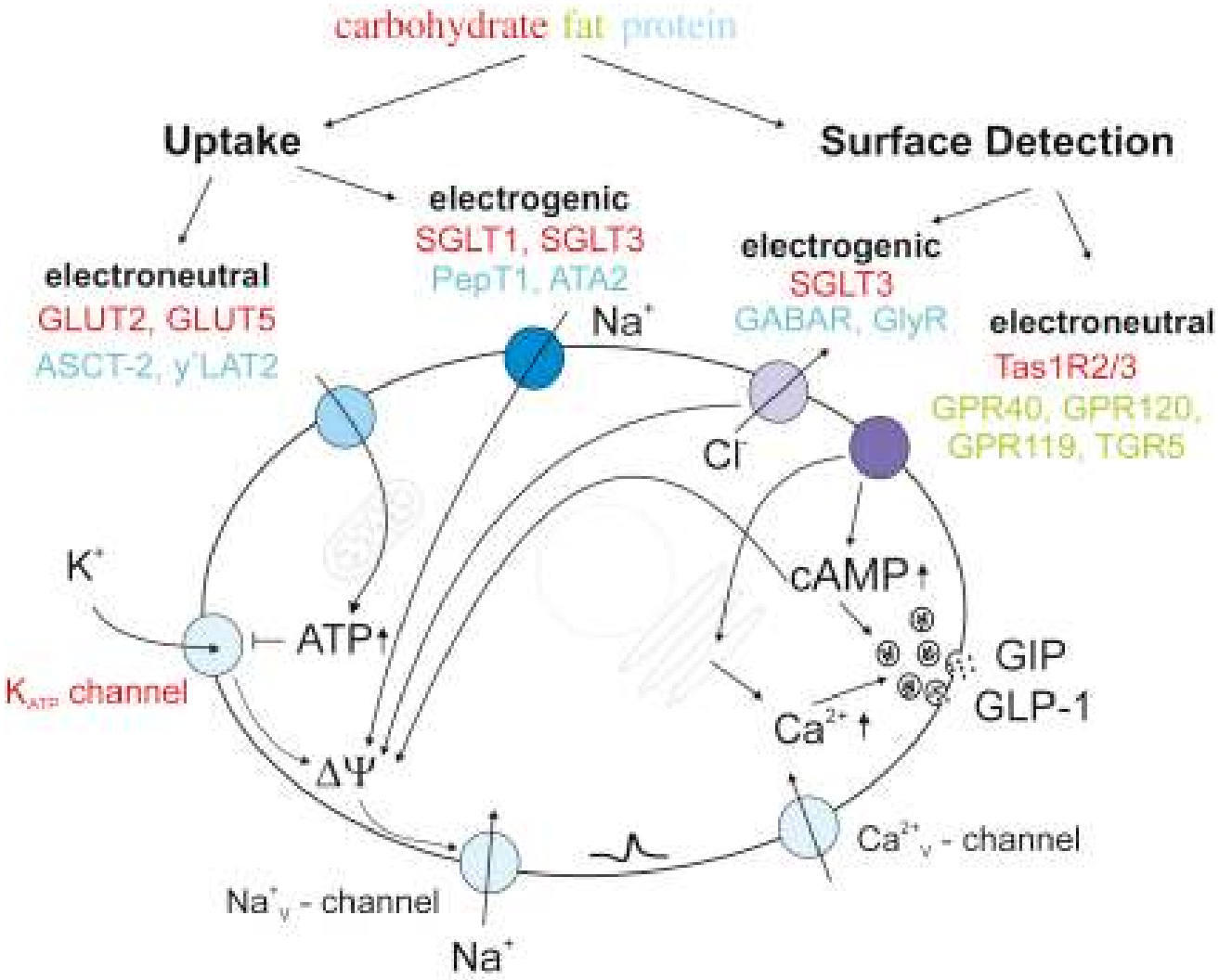
Among various monosaccharides, Glucose absorbed from the diet acts as the major signals to endocrine pancreas for secretion of Insulin, the chief hormone involved in energy homeostasis (glucose homeostasis). Glucose along with certain lipids and proteins also stimulate, enteroendocrine K cells and L cells in the intestine to secrete incretins - the peptide hormones , Glucose - dependent Insulinotropic Polypeptide (GIP) and Glucagon - Like Peptide-1 (GLP-1) respectively. These incretin hormones play important role in glucose homeostasis.

The arrival of macronutrients in the gut are sensed by, similar molecular sensors in K and L cells. The electroneutral uptake of nutrients are metabolized, increasing cytosolic ATP which closes ATP sensitive K channels leading to depolarization of cell membrane. As the calcium dependant voltage channels are opened, cytosolic Ca^{2+} levels are elevated resulting in incretin hormone release[96]. Nutrients also interact with cell surface sensors without entering the cell and triggers hormone release [56]. (Fig:1)

There are many gut factors and hormones that leads to enhanced insulin secretion after a meal and they constitute ,Enteroinsulinar axis, which was first coined by Ungar and Eisentraut -1969[40,41] .They maintain the glucose homeostasis by modulating the

hormonal output of endocrine pancreas against the circulating blood glucose concentration via the neural, hormonal and the circulating nutrient metabolites .

Fig 1 : Release of Incretin Hormones in GIT



Neural component of enteroinsulinar axis:

Neural component is said to have a significant role though little is known about it. Pancreatic exocrine and endocrine cells are subject to cholinergic, adrenergic and peptidergic innervations. Cholinergic innervations is responsible for " cephalic phase " of insulin release that is independent of nutrients absorbed where it regulates basal insulin secretion and postprandial insulin secretion (Ahren et al. 1986)[57]. Peptidergic neurons that innervate pancreas contains gut peptides like VIP-Vasoactive intestinal peptide and CCK-cholecystokinin acts as neurotransmitter for insulin secretion but its precise role is not eluded.

Nutrient components on enteroinsulinar axis:

The only dietary sugar, to stimulate insulin secretion through enteroinsulinar axis is Glucose. Glucose also has a crutial role in modulating insulinotropic potency of other stimulators on pancreas to exert its action[57]. Of the several amino acids that shows direct effect on pancreatic islet cells, arginine, lysine and leucine are found to be most potent stimulators[57]. A modest stimulatory effect is exerted on β-cells of pancreas by certain fatty acids and ketone bodies in the presence of Glucose (Gerich et al)[57].

Hormonal component of enteroinsulinar axis:

The intestinally derived incretins, GLP-1 and GIP are the two major relevant insulinotropic incretin hormones[42] that constitutes the hormonal component of enteroinsulinar axis.

Action of Glucose - dependent Insulinotropic Polypeptide (GIP):

GIP exerts glucose dependant stimulatory effect on insulin secretion in β cells of pancreas and a prompt insulin mediated uptake of glucose in various tissues. It is also found to inhibit gastric acid secretion and gastrointestinal motility at supraphysiological doses (Brown et al., 1975)[58].

Action of Glucagon like peptide -1 (GLP-1) :

The incretin hormone GLP-1 carry signals for pancreatic function, gastric emptying, intestinal motility and food intake (Willms et al., 1996). It exerts a potential insulinotropic effect by stimulating glucose dependant insulin secretion in β cells of pancreas. GLP-1 incretin consequentially regulates synthesis and secretion of insulin and glucagon in pancreatic islet cells. A proper ratio of insulin to glucagon is found to be regulated by GLP-1, which is essential for the regulation of carbohydrate metabolism and maintain glucose homeostasis. The ratio increases in postprandial state favoring anabolism and decreases in fasting state favoring catabolism.

Thus, after the digestion of macro and micro molecules in the diet, the major monosaccharide – glucose along gut hormones, signals pancreas for insulin secretion and

regulates carbohydrate metabolism and glucose homeostasis which is very much essential for every tissue in the body.

Overview of Blood glucose regulation in fasting and fed state:

In Fasting state:

Both insulin and glucagon, along epinephrine, nor-epinephrine[59] and cortisol maintains blood glucose concentration in the fasting state by upregulation of glycogenolysis, gluconeogenesis, lipolysis, and ketogenesis in various stages and tissues.

After a meal, glucose level reaches a peak in 1 hour and returns to fasting level in 2 to 4 hrs. During first 8 – 12 hrs of fasting the primary mechanism regulating blood glucose levels is liver glycogenolysis by hormone **glucagon** [60,61]. Even in fasting state a basal level of insulin is secreted to control glucose disposal but this low level of **insulin** in the fasting state has very negligible suppression action on glycogenolysis. After 12 hrs of fasting (overnight fasting) system enters a basal state or postabsorbtive state [62].

When most of glycogen were consumed, gluconeogenesis begins to maintain blood glucose levels and by 24 hrs of starvation when body glycogen stores gets depleted, gluconeogenesis becomes the main source of blood glucose. In gluconeogenesis, new non-carbohydrate precursors like lactate from muscles and RBCs, glycerol from adipose tissue and major amino acids (alanine and glutamate) from muscles are converted to glucose. Low insulin level suppresses lipogensis and activates lipolysis in adipose tissue and release free fatty acids. Muscles does not depend on insulin and

prefers free fatty acids as major fuel during rest and contraction sparing glucose for other tissues.

During prolonged starvation, a state of stress and very low level of plasma insulin results in epinephrine release. Epinephrine and Glucagon activates phosphorylation of hormone sensitive lipase and perilipin precipitating lipolysis [63]. By 3 days of starvation acetyl coA gets accumulated in the liver and drives the ketone body formation that meets the energy need. Thus, a regulated insulin to glucagon ratio is essential to maintain blood glucose level in fasting state.

In fed state:

After a meal, there is a postprandial glucose flux, that maintains a balance between glucose appearance and glucose disappearance or its uptake in the circulation. Glucose appearance is regulated by pancreatic and gut hormones (both hepatic and meal derived glucose production) and glucose disappearance regulated by insulin mediated uptake in the peripheral tissue (Pehling et al.),[64,3]. Various glucoregulatory hormones are insulin and amylin from beta cells , glucagon from alpha cells , GLP-1 and GIP from enteroendocrine cells.

In fed state, plasma glucose is increased by ingested nutrients that leads to Insulin secretion and synthesis ,which are enhanced by gut hormones. Insulin signals insulin sensitive peripheral tissues for glucose uptake and promotes glycogenesis in the liver. The secreted endogenous insulin along with amylin from beta cells of pancreas

inhibit postprandial Glucagon secretion where Insulin acts via paracrine action and amylin via centrally mediated efferent vagal signals [65] and stops glycogenolysis and gluconeogenesis in the liver. Thus insulin acts to reduce blood glucose level[3]. Insulin also has important role in activation of lipogenesis, storage of triglycerides in adipose tissue, protein synthesis in liver and muscle tissues and also cell growth and proliferation[66].

In T2DM, the earliest metabolic abnormality detected was insulin resistance that initially augmented insulin secretion to offset the defect in insulin action and maintains normal glucose tolerance (NGT)[67]. However overtime when beta cells begins to fail, leads to deterioration in glucose homeostasis and ultimately develop T2DM, which initially manifests with impaired glucose tolerance and latter as overt diabetes [67].The emerging hyperglycemia with poor metabolic control leads to glycotoxicity and further declines insulin sensitivity[67] progressing to other complications.

Sufficient and regulated insulin secretion is particularly needed to maintain glucose homeostasis both in fasting and fed state.

Insulin :

The primary function of β-cells of pancreas is synthesis, storage and regulated secretion of insulin. Insulin is a major polypeptide hormone secreted by pancreas. Insulin is made of A chain with 21 aminoacids and B chain with 30 amino acids. Insulin gene is located on the chromosome 11p 15.5.[29] .Three exons and two introns are in this gene.

Processing of insulin granules :

The messenger RNA after splicing codes for the preproinsulin peptide[30]. The signal peptide of preproinsulin translocates it from cytoplasm to lumen of rough endoplasmic reticulum where the signal peptide is removed and proinsulin precursor molecule is generated[31].The newly synthesized proinsulin gets accumulated in clathrin-coated region of the trans- Golgi network, where secretory granules are processed[32]. During the maturation of secretory granule, proinsulin precursor molecule is processed to active insulin. Thus in the intracellular storage pool of β- cells, mature β granules made of insulin in crystalline form along with soluble C- peptide are stored. After receiving the intracellular signals, insulin and C-peptide are released from secretory granules by exocytosis[33].

Pancreatic β – cell - insulin granule pool:

In β- cells insulin containing granules are either free in cytosol as reserve granules or docked to the plasma membrane [34].These docked granules may be primed or unprimed. The primed granules may be in readily releasable group or immediately releasable group.[35]

Insulin secretion:

Insulin release primarily determined by blood glucose levels. The number of insulin granules released from β-cells of the pancreas is also determined by blood glucose concentration. Insulin release is biphasic. The two phases in glucose stimulated insulin secretion are:

First phase of insulin secretion:

As the blood glucose level increases, glucose is rapidly taken up and metabolized by β cells of pancreas via glucose transporter 2 (SLC2A2/GLUT2). By glucokinase ,glucose is converted to glucose 6 phosphate that completes glycolysis and enters Krebs cycle resulting in increased cytosolic ATP concentration within 1 min (Porterfield et al. 2000). This leads to closure of ATP-sensitive potassium channels (K^+_{ATP}) followed by depolarization of cell membrane (Henquin, 1990). Depolarization causes opening of voltage dependant Ca^+ channels. As the [Ca^{2+}] concentration increases exocytosis of insulin is triggered.(Fig : 2)

In the first phase of insulin release, granules in the immediately releasable pool are released first. Number of granules released depends upon glucose concentration followed by changes in depolarization, Calcium channel activation and increase in Ca^{2+} ion concentration in the β- cells [36].As Ca ion concentration increases the rate of exocytosis of immediately releasable insulin granules increases. Hence depolarization of ATP – sensitive potassium channels results in increased Ca^{2+} entry and stimulate insulin release[37].This is known as KATP channel – dependant signaling pathway[38].The immediately releasable granules makes up the first phase of insulin secretion and the size of this pool is eminently regulated by time dependant potentiation (TDP) and by signaling molecules like Glucogon like peptide -1 and Pituitary adenylate cyclase - activating polypeptide (PACAP). (Fig :3)

Fig 2 : Glucose Induced Insulin relase

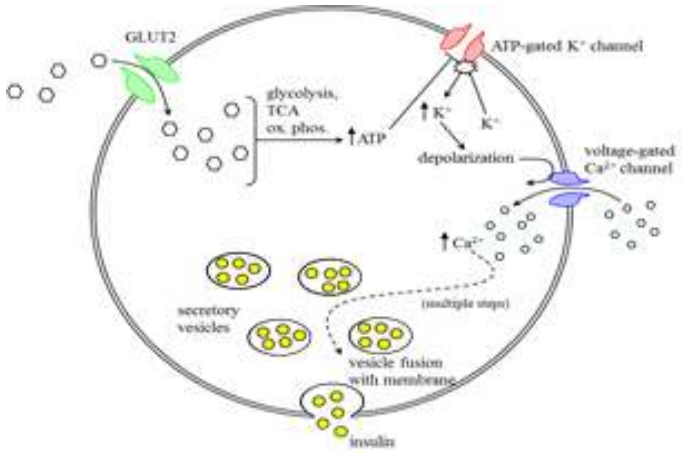
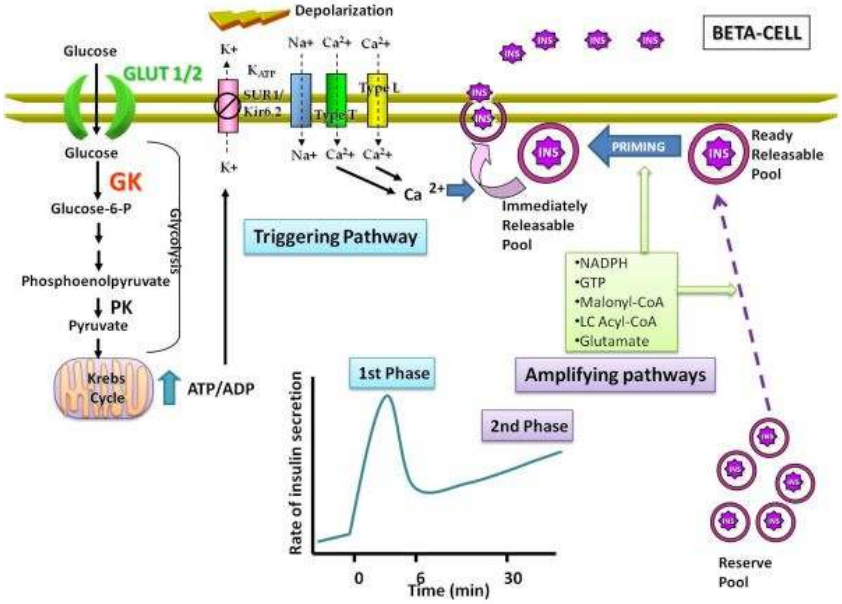


Fig 3 : First and Second phase of Insulin Release



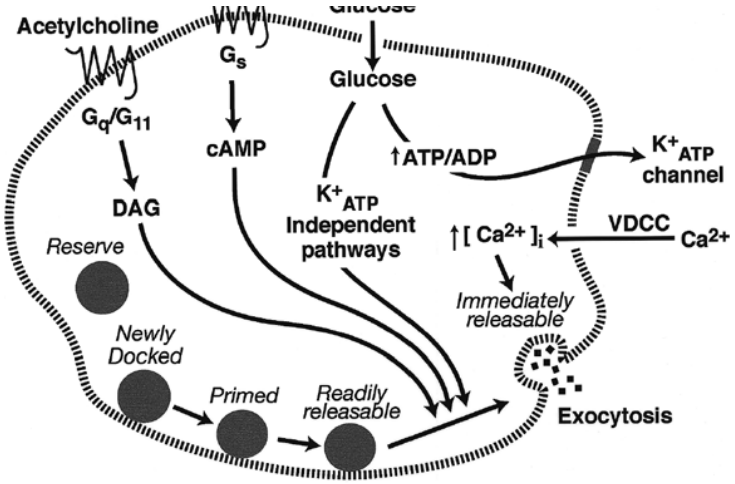
Second phase of insulin secretion:

Once the immediately releasable granules has been discharged, the size of this pool decreases and it is replenished by rapid transformation of readily releasable granules by agents like Glucogon like peptide-1, Pituitary adenylate cyclase - activating polypeptide (PACAP), acetylcholine and cholecystokinin. As the GLP-1 signal increases, it significantly alters the size of immediately releasable granule pool by increasing the size followed by insulin exocytosis. This is known as KATP channel – independent signaling pathway.

Thus the two pathways act synergistically. KATP channel – dependant signaling pathway triggers insulin release by increasing Ca^{2+} and KATP channel – independent signaling pathway enhances the response to increased Ca^{2+}. It is found that even in absence of extracellular Ca^{2+} or increase in intracellular Ca^{2+},KATP channel – independent signaling pathway can enhance insulin release. [39]

There exist a state of equilibrium between readily releasable and immediately releasable pool of primed docked granules. This depends on ambient glucose concentration, activity of KATP – channel independent signaling pathways and effect of time dependant potentiation. Activation of KATP – channel - independent pathways and TDP drive the equilibrium bar towards the right thus it increases the size of immediately releasable pool. Low ambient glucose concentration as occuring during fasting drives the equilibrium bar towards the left and the size of immediately releasable pool of the first phase of insulin release is decreased.(Fig :4)

Fig 4 : K^+ ATP – Dependent & Independent pathways



Release of insulin from beta cells of pancreas mainly depends on blood glucose concentration. Glucagon like peptide -1 (GLP-1) an efficient incretin hormone found to potentiate insulin synthesis and secretion from beta cells of pancreas through the enteroinsulinar axis depending upon the blood glucose concentration. Hence significant amount of potentially efficient GLP-1 secretion is essential for insulin release in the postprandial state .

In intestinal L cells, the gene encoding GLP-1 is Proglucagon gene. The Proglucagon gene is not only expressed in intestinal cells but also in pancreatic cells and brain cells. According to Darnell in 1982, stated that during gene expression a diversification of genetic information can take place at one or more levels like

1)duplication and divergence of gene ,

2) RNA precursors undergoing alternative splicing and

3) polyproteins undergoing alternative processing [48].

For Proglucagon gene tissue specific differential post – translational processing of proglucagon polyprotein determines different products in various tissues [48] .

Proglucagon gene structure:

In the whole mammalian species , a single unique gene encodes Proglucagon and it is located in chromosome 2q36-37. Proglucagon gene revealed a complex structure. The entire nucleotide sequence of human proglucagon gene spans nearly 9.4 kilobases

that includes 6 exons and 5 introns [37] into which 5'flanking region along the first exon are also included. Exon include the sequence that code for amino acids of specific protein and also untranslated sequence that are highly essential for efficient translation of mRNA transcript of Proglucagon gene. All 5 introns matches the consensus splice sequences of nucleotide at 5' end (GT) and 3' end (AG).

Exon 1 encodes 5' untranslated sequence completely but for 9 nucleotides. Exon 2 encodes part of Glicentin related pancreatic peptide (GRPP) with the signal peptide. Exon 3 encodes remaining part of GRPP along complete Glucagon sequence. Exon 4 encodes GLP-1 and exon 5 encodes GLP-2. Exon 6 encodes 3' untranslated region along last 4 nuleotides of coding region. After sequencing nearly 600 nucleotides of

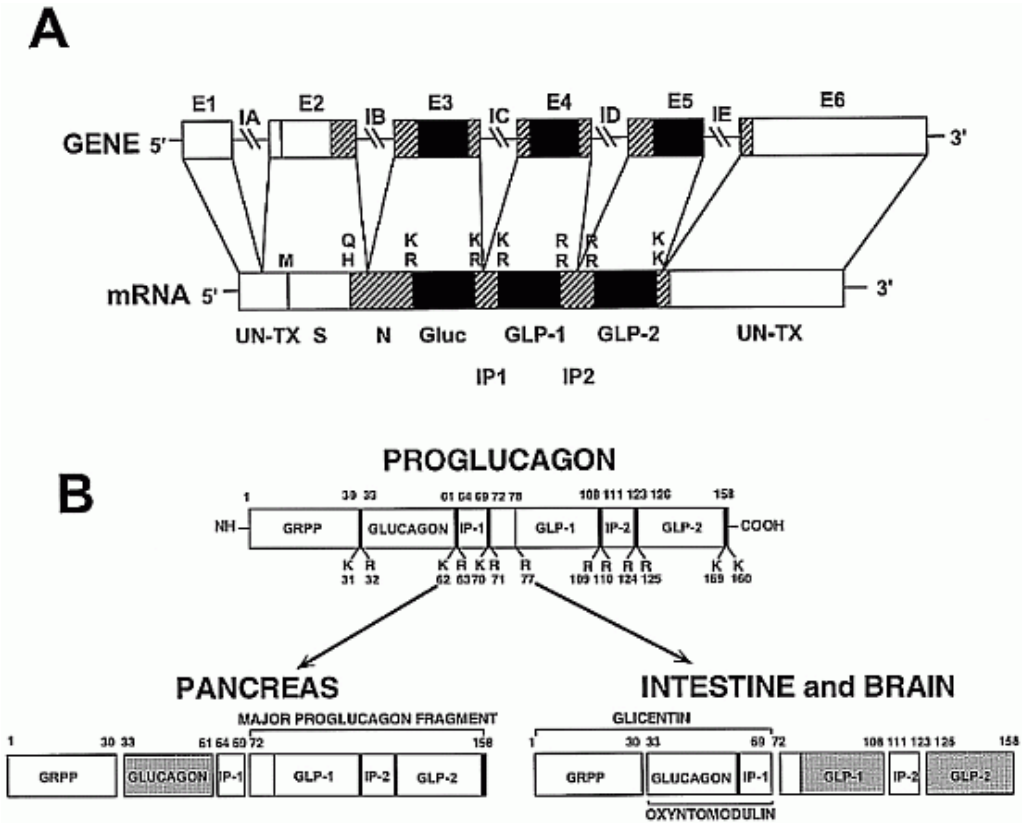
5'flanking region, TATA box (the core promoter sequence that forms preinitiation transcription complex) was detected from nucleotides -24 to -19 base pairs from transcription start site and not any CAAT box was found in nucleotide -68 to -65 base pairs representing complement of the consensus CAAT sequence. The TATA box region in the gene sequence was found identical between species and in 5' flanking region of proximal 130 base pairs nearly 88% of nucleotide sequence are homologous between species (rat and human) also the CAAT sequence from -93 to -50 in the 44 nucleotide stretches were identical. This shows the extreme importance of the promoter region that controls transcription that is being highly conserved through evolutions[49]. Introns did not show any detectable similarities[49]. From such a complex Proglucagon gene, the mRNA transcript synthesized in pancreas, intestine and brain were very much identical.

Posttranslational processing of Proglucacon gene and Products:

For Proglucagon gene having such a complex gene structure, its mRNA nucleotide sequence was derived from human cDNA(Bell et al. 1983b) and from that the structure of precursor proglucagon was deduced. More than 90% of amino acid sequence were found homologous to other mammalian species [49]. The precursor Proglucagon having large molecular weight was found to made of 160 aminoacids. The precursor Progluacgon undergoes endoproteolytic cleavage in the process of post-translational modifications. The various proglucagon derived peptides (PGDPs) were Glucagon, Glicentin, Glicentin – related pancreatic polypeptide (GRPP), Oxyntomodulin,

Major Proglucagon Fragment (MPGF) including Glucagon – like peptide 1 and Glucagon like peptide 2 (Mojsov et al.,1986). Such a diversified expression of Proglucagon gene product is mainly by differential tissue specific post translational processing. This differential processing of proglucagon gene takes place in α cells of endocrine pancreas, enteroendocrine L of intestine and brain neuronal (hypothalamus and nucleus of solitary tract) cells. Each Proglucagon gene products exhibits specific functions that regulates blood glucose homeostasis, cell proliferation and satiety significantly.[47] (Fig:5)

Fig 5: Expression of Proglucagon Gene and its Products



Processing in pancreatic α cells:

In the α cells of pancreas Prohormone convertase 2(PC2) is coexpressed during the processing of proglucagon gene. By proteolytic cleavage by PC2, precursor

Proglucagon yields four fragments. The main products are Glucagon, Major Proglucagon Fragment, Glicentin related pancreatic peptide and an Intervening peptide.

1. Glucagon:

Glucagon is made of 29 aminoacids that corresponds to 33 to 61 amino acid position starting at the N terminal amino acid within the proglucagon peptide (PG 33-61). The glucagon sequence in proglucagon gene is flanked by pairs of basic amino acids that acts as cleavage site for specific bioactive peptide release. (Steiner et al., 1980)[48].

Glucagon is an important peptide hormone from α cells of pancreas. Glucagon has a major role in glucose homeostasis by opposing the action of insulin and activating hepatic glycogenolysis and gluconeogenesis (Lefebvre, 1983; Unger and Orci, 1981; Habener, et al., 1985). Glucagon is secreted when blood glucose level is low. But repeated episodes of hypoglycemia leads to impairment in counter regulation by deficient or absent glucagon secretion in response to hypoglycemia [50].

It is well known that in pancreatic α cells insulin inhibits proglucagon gene expression as glucagon is the primary counterregulatory hormone of insulin and it is a

physiological inhibitory response. In T2DM, either due to insulin resistance in α cells or low insulin level leads to dysinhibition of glucagon secretion after a meal[51].

2. Major Proglucagon Fragment :

MPF is a large unprocessed polypeptide of molecular weight approximately 10,000 that was secreted in parallel with glucagon[52].It corresponds to PG 72-158.

3. A Glicentin Related Pancreatic Peptide

GRPP corresponding to PG 1-33 and an intervening peptide1, a small fragment of proglucagon sequence corresponding to PG 64-69 were also secreted[50].

For Proglucagon gene expression in α cells of pancreas, transcription factors play a significant role that acts on the promoter sequence and initiates transcription of proglucagon gene. Insulin was found to directly inhibit proglucagon gene expression by regulating gene transcription[53]. Insulin regulates transcription via insulin responsive - DNA elements[68]. Insulin action were confirmed by interaction of Transcription factor Pax6 with proximal promoter and more distal enhancer - like element[69]. Other transcription factors that regulate proglucagon gene expression are Fox 01, isl-1,cdx-2/3, pax-6, HNF-3a, HNF-3b, and brn 4 [70,71].

Processing in Brain Cells:

In Central nervous system, Proglucagon gene are expressed in nucleus of solitary tract of brain stem that projects fibres to hypothalamus, arcuate nuclei and paraventricular nuclei. Processing of Proglucagon gene showed pronounced contribution

to Oxyntomodulin along GLP-1 and GLP-2 . Effect of GLP-1 in CNS is regulation of appetite (Turton et al., 1996; Gutzwiller et al.,1999; Verdich et al., 2001) and delaying gastric emptying (Schirra et al., 1997). It is found to regulate food intake and maintain nutrient homeostasis [48,72,73].

Processing in Intestinal L cells:

In the intestine, L cells are distributed more in the distal ilieum. During proglucagon processing Prohormone convertase 1 is coexpressed in L cells, that yields Glicentin, Oxyntomodulin, two Glucagon like peptides (GLP-1 and GLP-2) and an intervening peptides 2 (IP-2) [38] by Prohormone Convertase 1(PC1)[74].

1. Glicentin :

The amino terminal part of proglucagon gene yields Glicentin. It is made of 69 amino acids. It corresponding to PG 1-69. Exogenous Glicentin were administered and it showed stimulatory effect on small bowel growth in rodents [75]. Specific receptor for Glicentin are not found .

2. Oxyntomodulin :

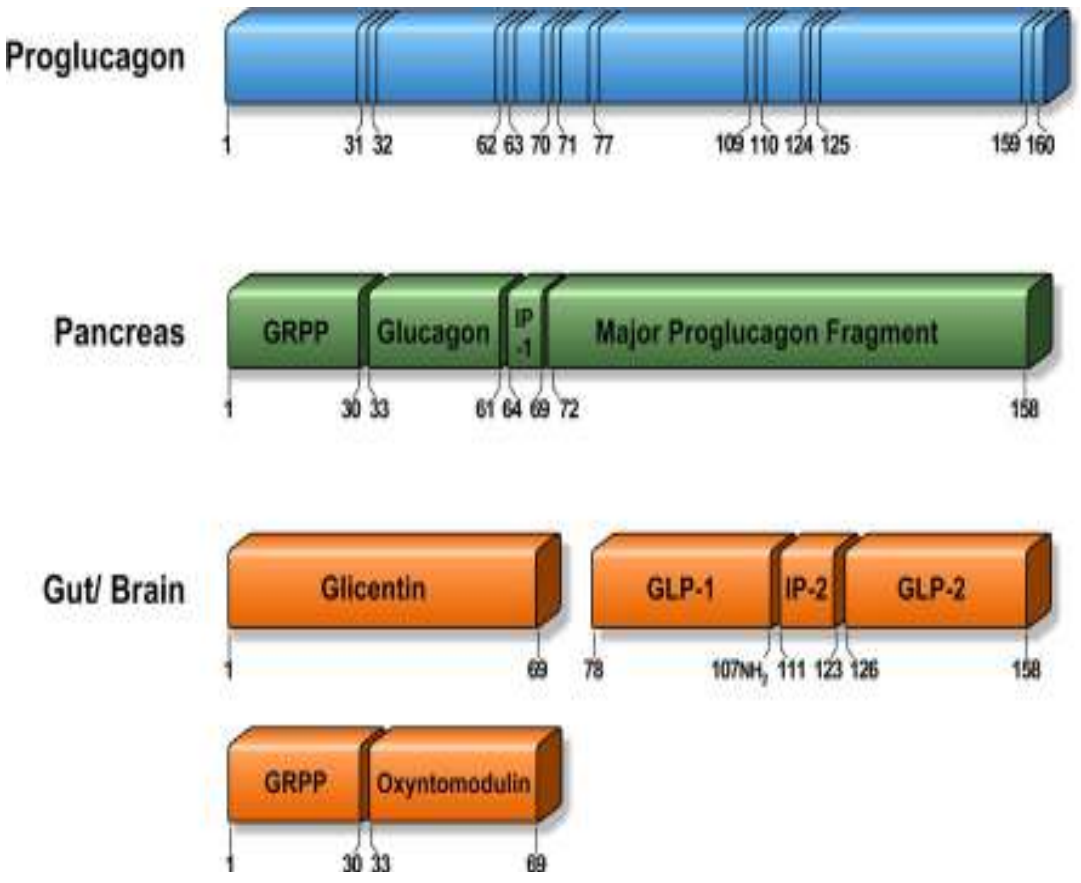
Glicentin peptide may either remain uncleaved or get partly cleaved to form Oxyntomodulin. It is made of 37 amino acids. It corresponds to PG 33-69. Oxyntomodulin has anorectic action by stimulating satiety and gastric acid secretion. It is used in antiobesity drugs (Murphy et al. 2006). It exerts its action through GLP-1R signaling system[76].

3. Glucagon like peptides :

The Major Proglucagon Fragment containing GLP-1 and GLP-2 peptides. It has a canonical cleavage sites made of pairs of basic amino acids flanking both the GLP sequences for the action of prohormone convertases 1 (PC 1). Proteolytic cleavage at this site yields GLP-1 (PG 72-108) and GLP-2 (PG 126-158)[53]. (Fig:6)

Fig:6 Proglucagon Gene Products in Pancreas, Gut and Bra

in



GLP-1 peptite structure:

GLP- is a peptide hormone. It exists in 4 isopeptides forms – GLP-1(1-37), GLP-1 (1-36), GLP-1 (7-37), GLP-1 (7-36). GLP-1(1-37) and GLP(1-36) amide have 37 and 36 amino acids respectively corresponding to PG 72-108. The two amino terminally truncated isopeptides GLP-1(7-37) and GLP-1(7-36) amide have 31 and 30 amino acids respectively corresponding to PG 78 – 108 [54].

The Amino terminally truncated forms of GLP-1 has distinct insulinotropic activity. GLP-1(1-37) may be called as prohormone having arginine in 6^{th} and histidine in 7^{th} position that by cleavage at single arginine residue at 6^{th} position yields GLP-1 (7-37) the active form (Lund et al., 1983)[47]. In this amino terminally truncated form the 108^{th} amino acid corresponding to glycine can get amidated with the carboxy terminal of Arginine residue.

Truncated GLP-1 exist in two equipotent forms as amidated GLP-1(7-36) or Glycine extended (7-37) form. In human gut most of the GLP-1 are secreted in amidated form GLP-1(7-36) having 30 amino acids. Amidated GLP-1 are more active form and shows slightly more resistance to plasma enzymes DPP-4[77]. GLP-1 in full length is inactive.

Stimulus for GLP-1 secretion in L cells:

Morphologically intestinal L cells and pancreatic α cells differ in the shape of granules where α cells have core of dense material surrounded by less dense halo and

L cell granules are homogenous without any halo[78]. The enteroendocrine L cells, known as open type endocrine cell have slender triangular base seated on the basal lamina with elongated cytoplasmic process with microvilli that open into gut lumen. Nutrients may be sensed by these microvilli and transmit the message for secretion of hormones[78]. Neural and hormonal signals from proximal gut were also found to be efficient indirect signals for GLP-1 secretion [50]. But when insulin is present in pathological concentration like hyperinsulinemic status and insulin - resistant status it shows a strong stimulatory effect on proglucagon mRNA expression and GLP-1 production in intestinal L cells Yi et al.,[79] eluding the fact that there exist a crosstalk between Wnt signaling pathway and insulin affecting the homeostasis of GLP-1 [79].

Metabolism of GLP-1:

Intact GLP-1 has a very short half -life of 1-2 minutes in circulation. It is rapidly degraded by enzyme Dipeptidyl peptidase 4 (DPP 4) and neutral endopeptidases. DPP4 is expressed on brush border of enterocytes and lamina propria of endothelial cells lining the capillaries[50]. The catalytic action of DPP4 cleaves off the two amino terminal amino acids of GLP-1 (cleaves at His7 and alanine 8) generating inactive GLP-1 (9-36) and GLP-1 (9-37) amides named as GLP-1m. Metabolic clearance rate of inactive GLP-1 by kidney is very rapid with a half life of 4-5 mins [80,81].

Stimulus for GLP-1 secretion:

Major amount of GLP-1 secretion is strictly meal dependant**[82]** even though a basal rate of secretion is maintained. GLP-1 secretion depends on the size of the meal

and in accordance to gastric emptying rate [83,84]. Along carbohydrates lipids also provide strong stimulus for GLP-1 secretion in L cells [85].

Actions of GLP-1 : (fig:7)

1. GLP-1 has main effect on insulin synthesis and secretion in β cells of pancreas.

2. **Effect on α cells of pancreas:**

   GLP-1 also has effect on α cells of pancreas. Glucagon secretion is strongly inhibited by GLP-1[54]. It exerts its inhibitory effect when glucose level is at or above fasting level. The inhibitory mechanism of GLP-1 on glucagon is not completely elucidate. Suppresion of glucogon release may be either directly by GLP-1 action or indirectly as paracrine action of insulin itself [42] [44] [45].

3. **Effect on δ cells of pancreas :**

   GLP-1 stimulates δ cells of pancreas and stimulate somatostatin release which produces a short loop negative feedback on insulin secretion.

The extrapancreatic actions of GLP-1 are

4. **Effects on Gastrointestinal Tract :**

   GLP-1 is known as a enterogastrone. It exhibits strong inhibitory action on gastrointestinal secretion and motility by inhibiting gastric emptying [86,54]. It has effect as 'ileal brake. It exerts " Ileal break effect" via afferent sensory neurons by regulating parasympathetic neurons[54].

5. **Effect in heart:**

GLP-1 shows cardio-protective and cardiotropic effects on heart. The inactive amide form GLP-1m exhibits protective effect on heart.(Choand Kieffer,2011)[87].

6. **Effect in Liver:**

The GLP-1 (28-36) a short peptide showed some beneficial metabolic effect in liver and pancreas by the study Liu et al.,.[87]It regulates hepatic glucose production by D'Alessio et al.,.[88]

7. GLP-1m , the degradation product of GLP-1 said to have insulinomimetic effects on cardiac function and hepatic glucose production.[89]

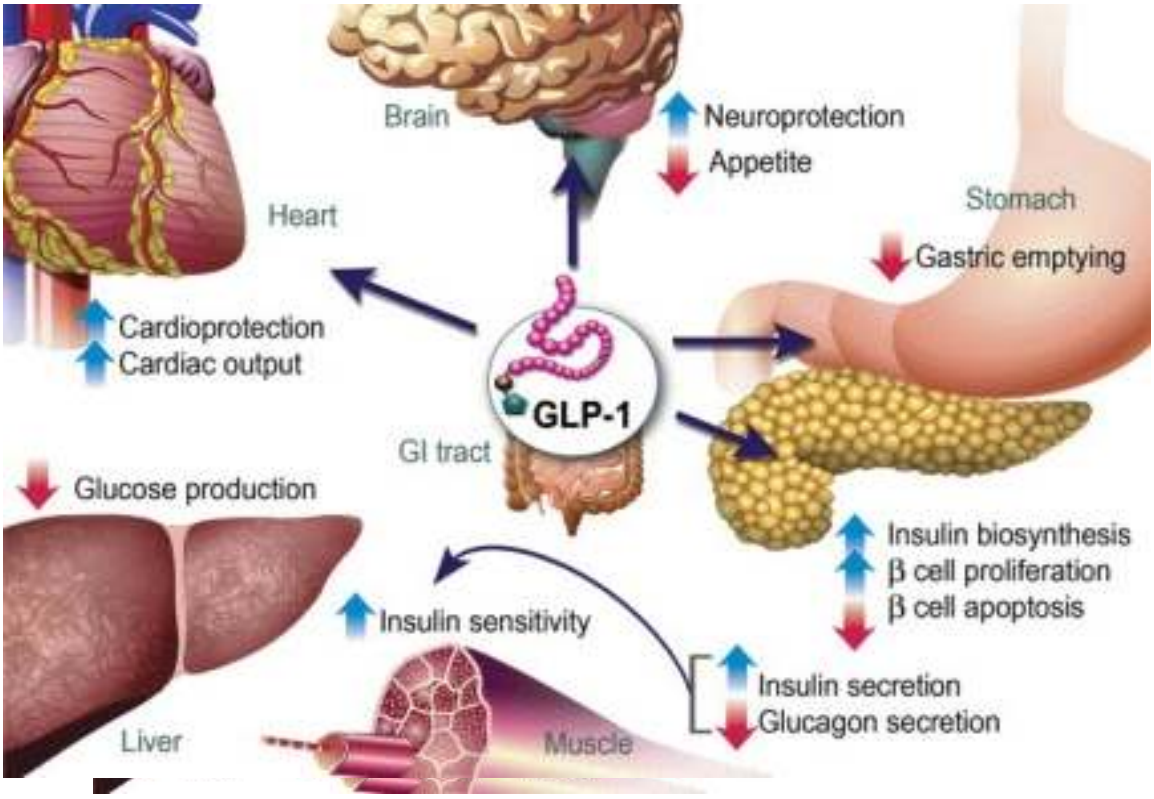
8. GLP-1 has effect on exocrine pancreas by inhibiting the secretion.

9. **Effect in Brain:** GLP-1 regulates satiety through GLP-1 receptors in brain .Its effect on appetite and food intake found to contributes to obesity partly.[89]

10. Physiological action of GLP-1 is found to increase peripheral insulin sensitivity[91].

11. During circulation in capillaries, interact with afferent nerve fibres emerging from nodosa ganglion and transmit signals to solitary tract and hypothalamus[54].

Fig:7 Functions of Glucagon like Peptide -1 (GLP-1)



Glucose homeostasis – regulation by GLP-1:

GLP-1 exerts its action through specific GLP-1 receptors. Human GLP-1 receptor gene spans 40 kb with nearly 7 exons is located in chromosome 6 (chr 6p21). GLP-1 receptor are made of 463 amino acids. It is a seven membrane spanning, class 2, heterotrimeric, G – protein coupled Receptor[95]. GLP-1 receptors are chiefly found in endocrine pancreas but also located in multiple tissues and cell type like central (brain stem, thalamus, hypothalamus) and peripheral nervous system, stomach ,duodenum ,heart, lungs, gastrointestinal tract, liver and kidney[91].

Mechanism of GLP-1 on β-cells of pancreas:

In β cells of pancreas, GLP-1 is an efficient direct stimulator of insulin release mediated by cAMP. In pancreas , GLP-1 also activates signal to proinsulin gene transcription through the transcription factor PDX-1[54], for biosynthesis of insulin [91, 49], stimulates signal – transduction pathways involved in proliferative and cytoprotective actions for maintaining mitogenesis and cell survival of β-cells .[50].

Action of GLP-1 on β cells of pancreas depends directly on the blood glucose concentration[50]. There exists a synergistic cross talk between glycolysis, on entry of glucose and cAMP, activated by GLP-1 signalling pathway. Thus β-cells require glucose, to respond to GLP-1 and GLP-1, to remain competent to respond to rising glucose levels. This mutual regulation between GLP-1 action and glucose metabolism is said as Glucose Competence Concept[43] . (Fig:8)

Fig:8 Glucose Competence Concept:

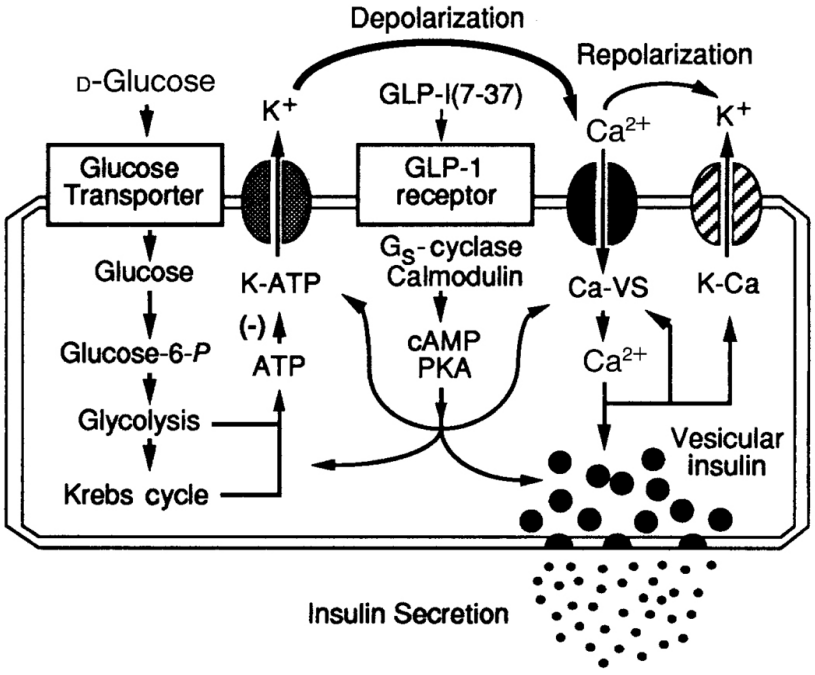
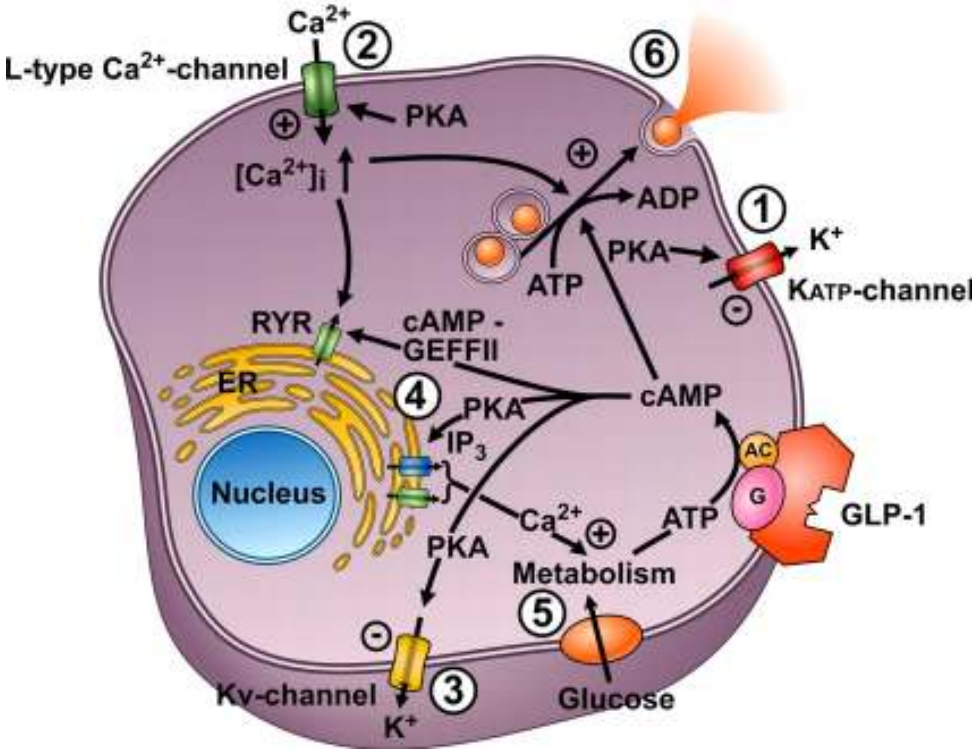


Fig: 9 Mechanism of GLP-1 induced Insulin Release



Cellular signaling through GLP-1 receptor is mediated by Gα and cAMP pathway[48]. GLP-1 binds to its G protein coupled receptor and activates adenyl cyclase that catalyses the conversion of ATP to cAMP, which acts as second messenger that allosterically activates PKA and cAMP –GEFII also called as Epac2 factor and enhances Ca^{2+} dependant insulin exocytosis (CIE)[91]. cAMP-GEF/Epac 2 factor activates rapid exocytosis of insulin granules and cAMP- PKA dependent pathway by prolonged depolarization shows slow exocytosis[94]. (Fig:9)

GLP-1 activates cAMP – PKA signals and blocks K-ATP channel, potentiating insulin release. These signals also increase L type VDCCs activity that enhances Ca influx leading to insulin release. Thus, cAMP –PKA dependant signals potentiates insulin granule mobilization[93] and enhances first phase of insulin secretion .

Epac2 factor is found to be involved in both phases of insulin secretion. GLP-1 activates cAMP mediated cAMP-GEF/Epac 2 factor regulates PKA - independent mechanism of insulin release[94]. For cAMP—regulated insulin granule exocytosis, a coordinated interaction of cAMP-GEF/Epac 2, Rim2, Piccolo and Rab3 takes place[94]. This coordinated complex is formed by specific interaction of cAMP-GEF/Epac 2 with nucleotide- binding fold (NBF)-1 of the SUR1 subunit of KATP channels. Insulin granule with putative SUR found to coupled with Cl^- channel (ClC3). As cAMP-GEF/Epac 2 binds to such SUR subunit, ClC3 channels are opened allows entry of Cl- ions into the insulin granules. Thus acidification of granules leads to priming of secretory granules and replenish the readily releasable pool[94]. GLP-1 activates Ryanodin receptor (RYRs) (Holz et al., 1999), on endoplasmic reticulum membrane of

pancreatic β cells through cAMP-GEFII/Epac2 for Calcium mobilization and potentiates insulin exocytosis [94].

Thus ATP sensitive K channels are closed by the synergitic action of GLP-1 and glucose. Induction of electrical activity due to depolarization leads to influx of Ca^{2+} ions. As the glucose and GLP-1 level increases, through PKA and cAMP-GEFII/Epac2 -dependent mechanisms $Ca2^{+}$ channels feeds forward into mobilization of intracellular stores of Ca^{2+}. Increased Ca enhances mitochondrial ATP synthesis and triggers insulin exocytosis that is continuously potentiated by cAMP.

Thus, cAMP was found to potentiate both first and second phase of Glucose induced insulin secretion where Ca^{2+} and cAMP interact cordially with each other. As glucose increases Ca entry, the Ca^{2+}/CaM complex activates Type VIII adenyl cyclase leading to increased cAMP level[92] which potentiates insulin release by both PKA dependant and PKA independent mechanism [94]. Quantitatively the ability of cAMP to increase the size of immediately releasable pool by accelerating granule mobilization is the most important one contributing more than 70% of total insulinotropic activity of GLP-1.[54]

Hence, GLP-1 found to have glucoregulatory, proliferative and cytoprotective action on β-cells of panceas.

GLP-1 in Type 2 Diabetes :

In recent research about development and treatment of T2DM, the main clinical interest is focused in GLP-1. Many studies postulated that T2DM may be due to

reduced or absent incretin effect[54]. With respect to GIP, secretion is normal or near normal but defect is mainly due to complete loss of insulinotropic effect[54]. But with GLP-1, its insulinotropic effect is completely preserved but a significant or sometimes substantial reduction in GLP-1 secretion is seen leading to T2DM[54].

Intestinal L cells secrete GLP-1 acts as a potent stimulator on beta cells of pancreas. It significantly increase insulin release along with insulin synthesis. GLP-1 also activates satiety center in brain regulating obesity. The pathway of GLP-1 synthesis, secretion and its action in various tissues is complex. Hence, defect in its pathway leads to various disorders of which its association with type 2 DM is more clinically specific.

In the mechanism of Proglucagon gene expression for the synthesis of GLP-1, many transcriptional factors have essential role. Of which transcription factor TCF7L2 was found to be one of the main initiator of promoter region of proglucagon gene through Wnt pathway leading to GLP-1 production. Many studies proved that TCF7L2 gene polymorphism which is found to alter this pathway, is associated with Type 2 Diabetes Mellitus, done in various populations.

Transcription factors affecting Proglucagon gene expression in Intestinal L cells:

Gene expression can be modulated in any step, from DNA-RNA transcription step till post translational modification of the proteins. The ProGlucagon gene encodes a large biosynthetic precursor and its expression in intestinal L cells is regulated at many levels and final products are GLP-1, GLP-2, Glicentin and Oxyntomodulin.

Proglucagon gene expression regulated at various levels are:

1. During transcription by transcription factors that shows tissue specificity on promoter region,
2. During RNA processing by alternative splicing,
3. By tissue specific post-translational processing.

Transcription of a gene in a DNA is initiated at the promoter region. During the exploration of Proglucagon gene in rodents and humans, a minimum G1 promotor region and four G2-G5 enhancer elements were identified in its promoter region. Various regulating factors of Proglucagon gene transcription were identified, they are cAMP, amino acids ,insulin hormone, Wnt signally pathway and many CAT/TCF like homeodomain (HD) protein transcription factors[97].

Isl-1, a transcription factor is found to regulate transcription of insulin gene, proglucagon gene and somatostatin gene in islet cells of pancreas that secrete various hormones [97].

Pax – 2 proteins were found to promote gcg transcription by binding to motifs between G3 and G1 enhancer elements in intestinal endocrine L cell, following an adenovirus - mediated overexpression method .[97]

Cdx-2, a caudal- like HD protein expressed in pancreatic and intestinal gcg-producing endocrine cells[97].

cAMP in proglucagon transcripiton in Intestinal L cells:

Proglucagon gene expression is activated by cAMP signals through CRE and other cAMP response elements. In both pancreatic α cells and intestinal L cells a typical cAMP response element (CRE) region at -291 and -298 bp within the proximal gcg promoter region, is recognized by cAMP signals, for Proglucagon gene expression .[97] A significant increase in mRNA expression and GLP-1 production is processed by either cAMP promoting agents or membrane permeable cAMP analogs (Drucker et al.1994) by activating this motif. But by deleting or mutating this CRE motif, only a partial repression was found on its stimulatory effect on gcg promoter expression[97].

The second messenger for cAMP is not only PKA but was also found to be by Epac-1 and Epac-2 also called as cAMP-GEFs). Epac molecules mediate its effect of cAMP by activating Rap-1-Raf-MEK-ERK signaling pathway. Expression of Epac -2 on intestinal endocrine L cells has been reported and found to be involved in the regulation of HD protein Cdx-2 through MEK-ERK signaling cascade for the gcg gene transcription and expression. (Chen et al. 2005)[97].

For the Proglucagon gene expression in the intestinal endocrine cells, the chief pathway is the Canonical Wnt signaling pathway and the key effector is the bipartite transcription factor β – Catenin/TCF7L2(Yi et al. 2005). Wnt gene along their receptors are expressed in pancreatic islet cells (Heller et al. 2003) and intestinal L cells epithelia [97].

Wnt signaling pathway:

Wnt signaling pathway was first identified during the research in breast cancer and the embryological studies in Drosophila, Xenopus. Wnt proteins are secreted as lipid

-modified signaling glycoproteins. In Wnt signaling these proteins act as ligands to activate many different Wnt pathways. Among Homosapiens nearly 19 Wnt ligands have been identified. They are Wnt1, Wnt2, Wnt2B, Wnt3, Wnt3A, Wnt4, Wnt5A, Wnt5B, Wnt6, Wnt7A, Wnt7B, Wnt8A, Wnt8B, Wnt9A, Wnt9B, Wnt10A, Wnt10B, Wnt11, Wnt16. The Wnt ligand found in intestine is Wnt-9b/14b/15 (Bouillet, P. et al.,). In Wnt – 9b 357 amino acid precursor with the signal sequence constituting 22 aa and a mature segment of 335 aa. There are 24 cysteines in mature segment that under goes palmitoylation and initiates targeting of Wnt ligands to specific receptors. The two categories of Wnt pathway are canonical and noncanonical . The three established Wnt signaling pathways are

1. Canonical pathway in which protein β- catenin is involved,
2. Non-canonical planar cell polarity pathway and
3. Non-canonical Wnt/Calcium pathway.

Non – canonical pathways operates independent of β-catenin[98].

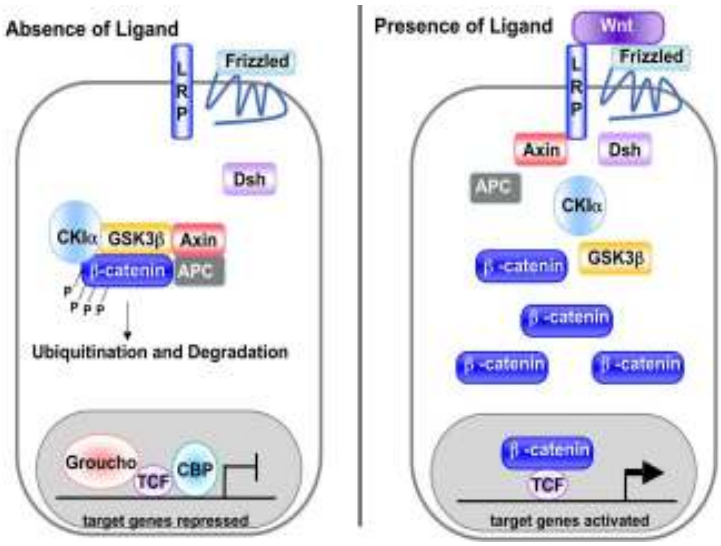
The Canonical β- catenin signal pathway or Wnt/β-catenin pathway:

Wnt receptor :

Wnt lipoglycoproteins binds to receptors of Frizzled family (Fz) and many coreceptors like lipoprotein receptor-related protein (LRP) – 5/6, Receptor tyrosine kinase (Ryk), ROR2. In humans 19 Wnt family proteins and 10 Frizzled receptors were identified. All members in Frizzled family receptor are similar. Frizzled receptor is a G protein coupled receptor has divergent N-terminal signal peptide, highly conserved

extracellular cysteine rich domain (CRD), seven transmembrane spanning domain, variable length linker region and variable C terminal region. Proglucagon gene transcription is mediated by Wnt/β-catenin pathway. In its pathway Wnt ligand binds to CRD region of Frizzled receptor with coreceptor as LRP-5/6. Thus, Wnt/Frizzled signal cascade is activated[99].

Fig:10 The Canonical β- catenin signal pathway or Wnt/β-catenin pathway:



The activated Frizzled receptor sends Wnt signals to the phosphoprotein Dishevelled (Dsh/Dvl) present in the cytoplasm by phosphorylation or kinases . Dsh protein has a highly conserved protein domains- an DIX amino terminal domain (Dsh and Axin), a PDZ central domain (Postsynaptic density-95,Discs-large, Zonula occludens-1) and a DEP carboxy terminal domain (Dsh ,Egl-10 ,Pleckstrin). These different domains of Dsh protein are significant determining factor in tranducing Wnt signals into three separate branches– the Canonical, non-canonical and Ca^{2+} pathways. In Canonical

pathway, Wnt signaling uses DIX and PDZ domains of Dsh to stabilize cytosolic β catenin[100].

In the absence of Wnt signal, the transcriptional coactivator β-catenin is not allowed to accumulate and is degraded by a multiprotein "destruction complex". The complex of proteins in destruction complex are tumor suppressors Axin and adenomatous polypoisis coli (APC), the Ser / Thr Glycogen synthase Kinases (GSK-3β), GSK3- binding protein (GBP), Casein Kinases 1 (CK1),Protein phosphatase 2A (PP2A) and the E3- ubiquitin ligase (β-TrCP / β-transducin repeat containing protein)[100]. In the destruction complex, phosphorylation of CK1 at ser45 followed by phosphorylation of Ser33, Ser37 and Thr41 by Glycogen synthase Kinase (GSK3). This phosphorylation

generates a β- TrCP recognition site near the β catenin amino terminus, which requires scaffolding of the β catenin and kinases by Axin. Hence, β –TrCP mediated ubiquitinated β – Catenin is subsequently degraded by the proteasome(99).

In the presence of Wnt signal, there is immediate recruitment of Axin protein complex to the activated Wnt receptor followed by GSK-3 beta and CK 1 phosphorylating LRP-5/6 coreceptor. The Axin-1 complex translocation disrupts the process of beta- catenin phosphorylation and degradation. Thus the binding of Wnt ligands to the cysteine – rich domain of Frizzled receptors resulted in disassembly of the destruction complex containing axin, APC and GSK3 leading to stabilization of β catenin. Beta catenin gets accumulated and is eventually imported into the nucleus where it serves as transcription activator of the TCF/LEF-1 family of DNA binding proteins[99].

Wnt signals have a wide range of fundamental cellular processes such as stem cell maintenance, embryonic development, cell fate, cell proliferation, cell migration, tumour suppression and oncogenesis[101].

In Wnt signaling pathway ,the TCF factor binding motif is located within the G2 enhancer element in the gcg promoter region. This region was already demonstrated that was found to mediate the effect of cAMP and Calcium on gcg promoter expression. Then it was proposed that there could be a crosstalk between Wnt signaling pathway and the cAMP – PKA signaling in the intestinal endocrine L cells (Yi et al. 2005). More recently evidences support the existence of crosstalk between insulin and Wnt signaling pathway, when a pathological dose of insulin given, it utilised the same elements in the gcg promoter region and stimulated gcg mRNA expression and GLP-1 production (Yi et al. 2008). (Fig:10)

TCF7L2 is an important transcription factor that regulates GLP-1 production. Before going in to the effects of TCF7L2 polymorphism, a short review about TCF/LEF family.

TCF/LEF family:

T–cell factor (Tcf-1) and Lymphoid enhancing factor-1 (Lef-1) are the founding members of the TCF family. [28] **TCF/LEF** family is a group of transcription factors that is involved in Wnt signaling pathway by binding to DNA through high mobility group box (HMG box). These transcription factors mediate Wnt signal pathway by recruiting co-activator beta catenin that enhances the elements of the gene or by recruiting co-repressors Groucho family members that represses the target gene

transcription. In a specific cell type if Wnt signal is absent, the promoter DNA shows a high level of DNA methylation and also repressive trimethylation of lysine 27 in histone H3 (H3K27me3). Hence if Wnt signals are absent, Wnt/β-catenin target genes become non-responsive and shows absence of active histone modification and Tcf/Lef proteins[102]. The apparent inability to cope with chromatin uncovers an intrinsic property of TCF/LEF proteins that prevents false ectopic induction and ensures spatiotemporal stability of Wnt/β – catenin target expression [102].

Human members of TCF/LEF family and Genes encoding are

1. TCF7 (TCF-1) - Tcf7
2. TCF7L1 (TCF-3) - Tcf7l1
3. TCF7L2 (TCF-4) - Tcf7l2
4. LEF. - Lef1.

Tcf/Lef genes show high tissue – specific expression patterns[102].For certain developmental processes individual Tcf/Lef genes shows unique requirement which if absent leads to loss of function [102]. The structure of Tcf/Lef protein family shows N-terminal β-catenin binding domain, sites for -related-gene (Grg)/transducin-like enhancer of split (TLE) transcriptional co-repressors, a high mobility group box (HMG-box) DNA binding domain and an adjacent nuclear localization signals[102]. Even though Tcf/Lef protein family show similar structure they are Promotor specific non-redundant proteins whose function differs with their domain composition[102]. Such a structural divergence is further enhanced by extensive and tissue specific alternative splicing in particular during the synthesis of Tcf7 and Tcf7l2 proteins[102].

Tcf-1 is exclusively expressed in T lymphocytes and Lef is expressed in both T cells and early B cells [T1] shows Tcf-1 and Lef are essential for early T-cell development. They adopt a distinct genetic wring that activates the $CD4^+$ T cell fate and an identity to $CD8^+$ T cells. The effect of Tcf/Lef transcription factor in CD4 verses CD8 lineage choice is mediated by activation of transcription factor Th-POK. In cell type specific induction study when Tcf1 , Tcf3 along Tcf1E splice variant and Tcf4 expression were changed, it lead to loss/change in induction of T/Bra , Cdx1,Cdx2 and Sp5Wnt target genes showing that these transcription factors mediate Wnt induction of its target gene [(102)]. In a study β-catenin/Tcf-4 axis showed profound effects on astrocyte biology. It was found that β-catenin /Tcf-4 axis influence the basal level of HIV transcription in astrocytes contributing to the progressing neuroinflammation[(103)].

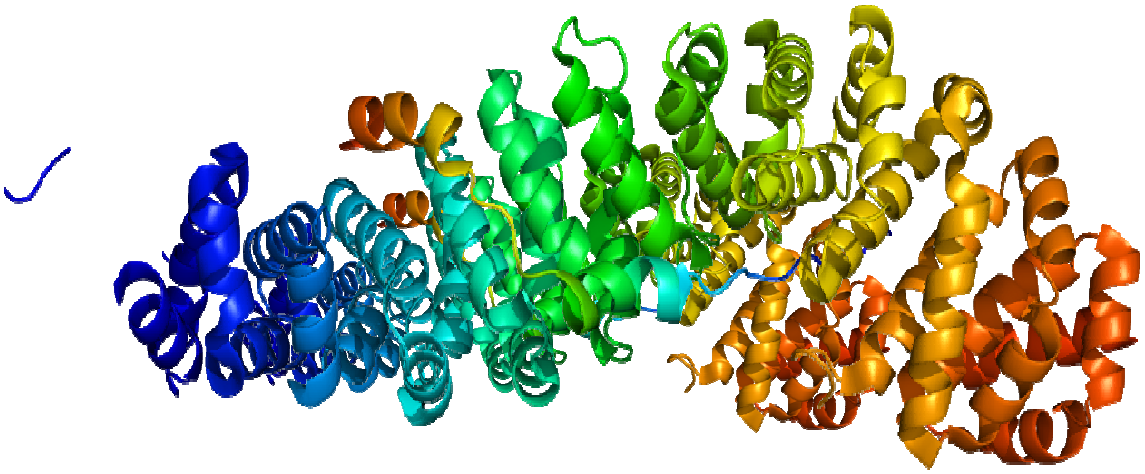
The Wnt signaling associated transcription factor TCF7L2 is expressed in many tissues including gut and pancreas. Many studies proved the effective β-catenin/TCF7L2 axis in initiating transcription at the proglucagon promoter region and GLP-1 incretin hormone synthesis[(104)]. For the action of GLP-1 on β cells it require TCF factor that β cell proliferation and insulin secretion [(104)]. TCF7L2 expression were down-regulated in adipose tissue of obese Type 2 Diabetic people showing the ineffective action of insulin due to insulin resistance [(104)]. Studies eluded that there is a cross-talk between insulin and Wnt signal pathway.

TCF7L2 / TCF4 Protein –Structure:

TCF7L2 means Transcription factor 7 – like 2 (T- cell specific ,HMG- box) gene . This gene encodes a high mobility group (HMG) box containing transcription factor .

It plays a key role in Wnt signaling pathway. Genetic variants of this Gene are found to be associated with risk of Type 2 Diabetes.

Fig: 11 TCF7L2



TCF7L2 is made of 596 amino acids (NP_110383.1). It has two conserved domains -

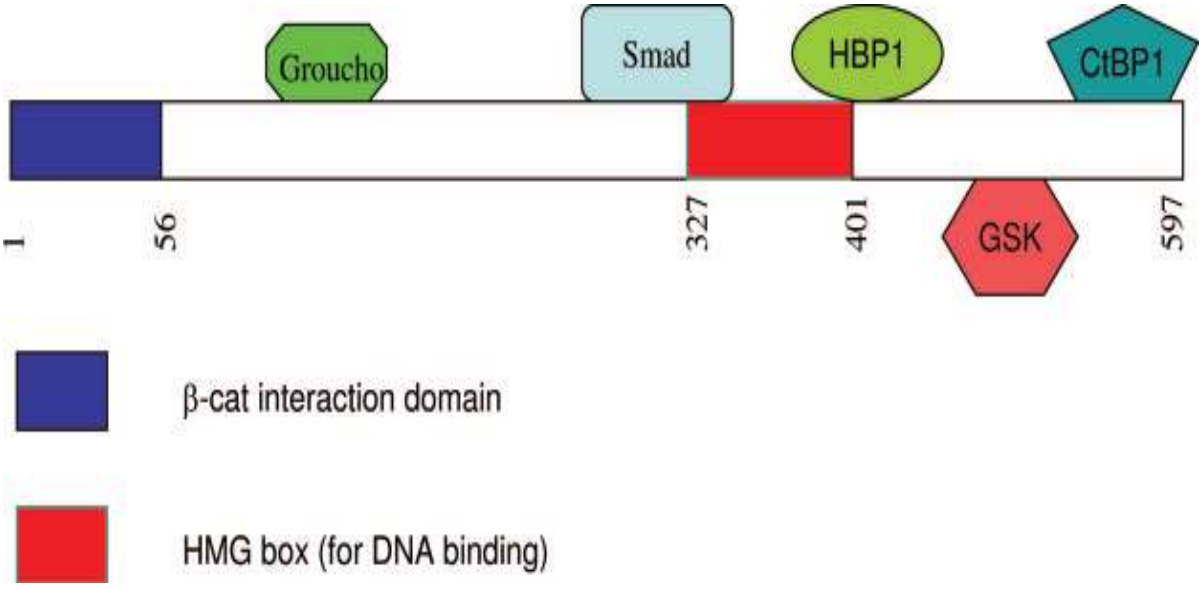
- the CTNNB1_binding domain and
- the SOX-TCF_HMG-box domain. (Fig:12)

The CTNNB1_binding domain is located from 1 to 236 amino acids. It is the binding site of β-catenin to form the active complex. The SOX-TCF_HMG-box domain is located from 330 to 397 amino acids. This domain binds to specific DNA motif and regulates gene transcription. The structure of TCF7L2 protein also shows a domain for Groucho protein. The amino acid sequences of HMG boxes of TCF7L1, TCF7L2 and TCF7 show striking homology.

In the absence of Wnt signaling, the HMG-box TCF proteins in the nucleus forms complex with Groucho and C-terminal binding protein 1 that inturn recruit

nuclear corepressors like Histone deacetylases (HDACs) and acts as transcriptional repressors to the promoter region of the Wnt target gene. In the presence of a Wnt signal where beta catenin is accumulated and diffuse into the nucleus . Interaction of beta catenin, DNA, Groucho and CtBP-1 to the functional domains of human TCF7L2 leads to activation of transcription of the promoter region of Wnt Target gene.[105]

Fig:12 TCF7L2 Domain Structure



TCF7L2 expression was found highest in Pancreas followed by colon, brain, small intestine, monocytes and lungs. Lowest expression in all tissue and no expression in T and B cells.

Expression and Functions of TCF7L2 gene in pancreatic β cells:

A comprehensive overview of the target genes for TCF7L2 in pancreatic islets is still lacking, but it is known to control expression of several genes involved in on cogenesis, such as c-jun, c-myc, Cyclin Dl, and many others. Shu et al [105] study showed that TCF7L2 has significant roll in β cell proliferation, β cell apoptosis and Glucose stimulated insulin secretion. TCF7L2 is involved in pancreas development and TCF7L2 along with SDF-1/CXCR4 transcription factors -induced cytoprotection of β cells. These two mechanism together increases β cell mass. But by TCF7L2 overexpression and TCF7L2 depletion it exhibits opposite effects in human pancreatic beta cells. This raises the question if TCF7L2 is a transcriptional activator and not a transcriptional modulator[105]. Finally in a silico study, they have identified that the

consensus sequence (WWCAAWG) for TCF binding is in the proprotein convertase 1 (PC1) and PC2 genes . Hence, TCF factor is essential for proinsulin conversion to mature insulin [105]. This is suggestive of TCF7L2 role in insulin processing, and if risk allele is present this process may be disturbed.

Expression and Functions of TCF7L2 gene in intestinal endocrine cells:

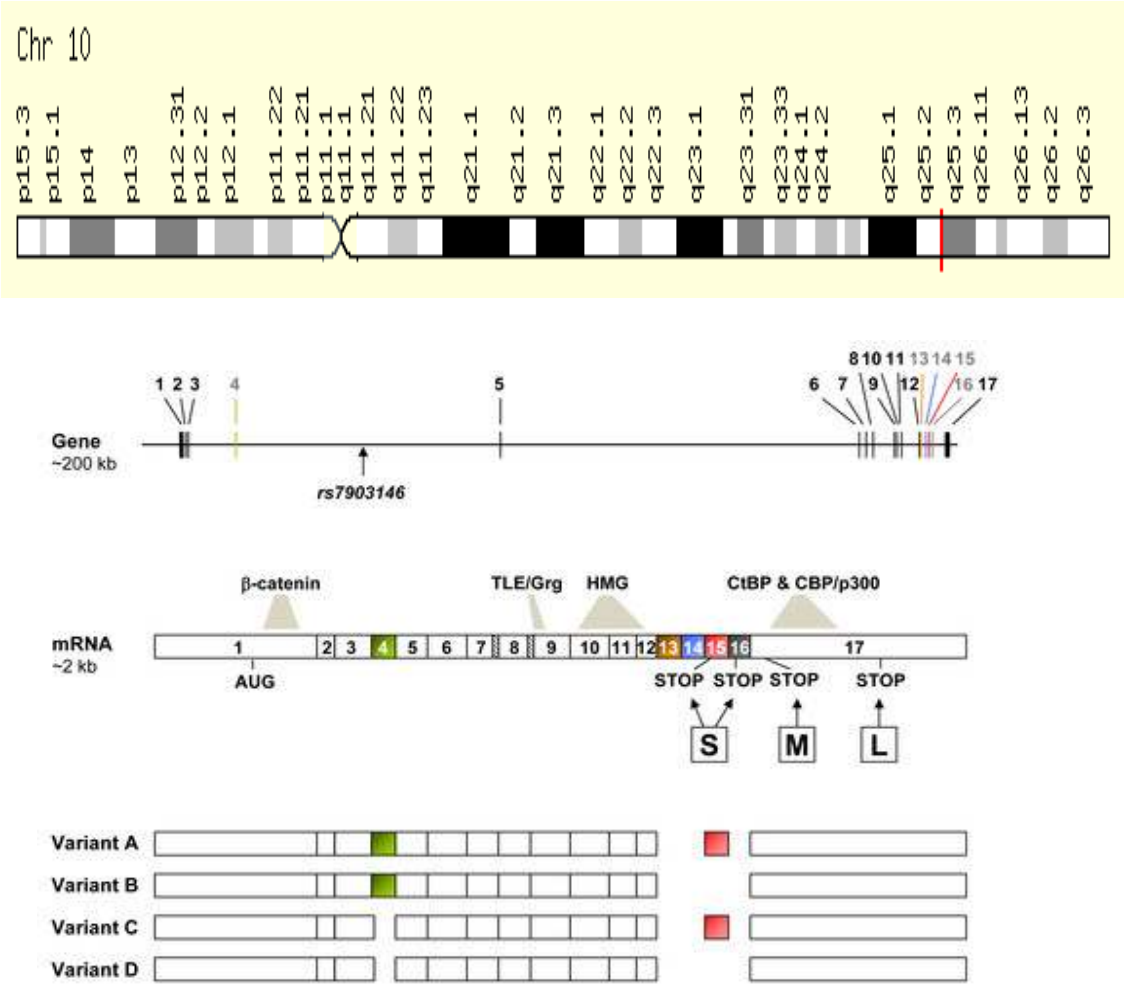
In the Wnt pathway, TCF7L2 a member of TCF/LEF family. is an important transcription factor that initiates transcription at the promoter region of proglucagon gene and express by GLP-1 secretion in the intestinal L cells[105]. Specific Wnt ligands stimulate TCF7L2 gene expression in L cells. TCF7L2 dependant signals were implicated in the oncogenesis of colorectal cancer [68,106] .

Recent studies identified oncogentic effect in breast cancer[68,107] and overexpresion of tCf7l2 cardiovascular diseases[68]. Studies showed the expression of TCF7L2 in human adipocytes.[105]

TCF7L2 gene :

The cytogenetic location of TCF7L2 gene is on the long (q) arm of chromosome 10 at position 25.2 to 25.3. More precisely the molecular location TCF7L2 gene starts from 114,710,009 base pair from pter and end at 114,927,437 basepair from pter. It is made of g 217,429 bases. (Fig:13)

Fig :13 Genomic Structure of TCF7L2



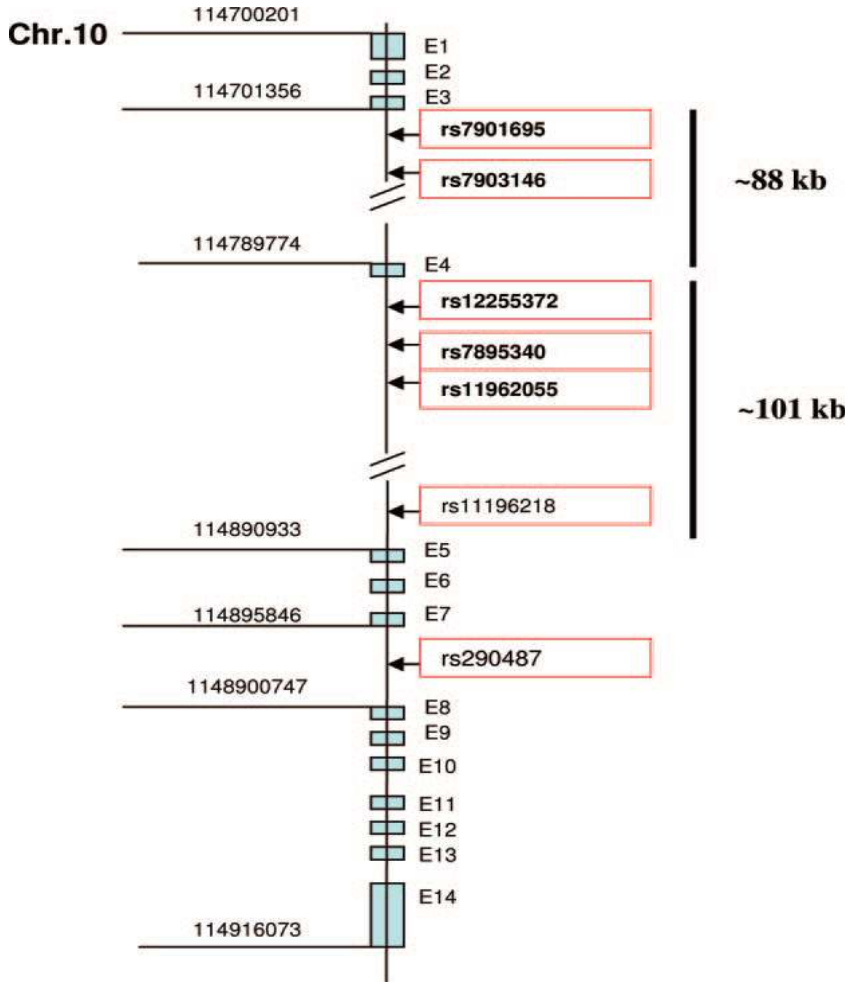
The Gene structure of TCF7L2 was determined by Duval et al.,(2000). It comprised of 17 exons of this 5 were alternative (i.e, exon 4 in 5' end and exons 13-16 in 3' end)[68]. Exon 14 and 15 are highly similar and exclusively tissue-specific so that exon 14 predominates in adipose tissue and muscle, whereas exon 15 predominates islets and lymphocytes.[68] Alternate splicing sites were identified in exons 7, 9, 16 and 17. Alternative transcriptional start sites in the promoter region and in exon 1 were reported and these are rare. This led to the synthesis of many TCF4 isoforms having short, medium or long C-terminal ends [68]. Such a variable exons results in different complex pattern of splicing and hundreds of potential protein isoforms are formed. This eluded the fact that alternative splicing leads to different TCF7L2 proteins isoforms that were found to either activate or repress the Wnt signaling pathway. How TCF7L2 variation affects the gene function is yet unclear .

Micro satellite are repeating sequences of 2-5 base pairs in DNA called simple sequence repeat or short tandem repeats. They are distributed throughout the genome and function as Molecular markers. Change in length of Microsatellite markers within promoter region leads to quick change in gene expression between generations.

Micro satellite markers within introns also influence the phenotype in various disorders like Friedrich Ataxia., Acute lymphoblastic leukemia , hypertension, osteosarcomas. Linkage disequilibrium is non- random association of alleles at one or more loci. In a randomly distributed genotype, the amount of disequilibrium depends on difference in observed allele frequency. When a combination of alleles and genotype are found in an expected

proportion then it is said to be in equilibrium. If a large block of linkage disequilibrium is found it is likely to be a resource for SNP[112].

Fig:14 The Diagram Shows the Positions of Seven SNPs and the Microsatellite DG10S478 in the Human TCF7L2 Gene



By Grant et al.(2006)[108],228 microsatellite markers were genotyped throughout 10.5Mb interval on 10q in Icelandic population with Type 2 Diabetes, a Microsatellite DG10S478 that was located within intron 3 of the TCF7L2 gene was found to be associated with risk of T2DM[108]. This study was replicated in Danish and US population. The TCF7L2 gene spans ~ 216kb. In this region, ~750 single nucleotide

polymorphism have been identified. The SNPs in the intron 3 region of TCF7L2 chromosome were at rs7901685, rs7903146, rs12255372, rs7895340, rs11962055, rs 11196218 and rs 290487. In an international HapMap project data, TCF7L2 was found to span several linkage disequilibrium [LD] blocks [main-03-20]. The SNPs associated with Type 2 DM forms the largest LD block which is spanning ~65kb. This LD block begins from middle of the intron 3 and ends at the first seventh of intron 4[108,109]. Specifically the two SNPs rs12255372 and rs7903146 were in strong linkage disequilibrium with Microsatelite DG 10S478 and this showed a robust association with T2DM[105]. Within Intron 3, the non-coding region show SNPs at rs12255372 and rs7903146 were found to be associated with T2DM . In another study between type 2 DM and controls, all exons of TCF7L2 gene were sequenced and it did not show any non-synonymous SNP (nsSNP). This excluded the possibility of protein sequence polymorphism leading to T2DM risk. Then it was postulated that the regulatory TCF7L2 genetic variation leads to change in its target gene expression and this increases the susceptibility for T2DM[108]. (Fig:14)

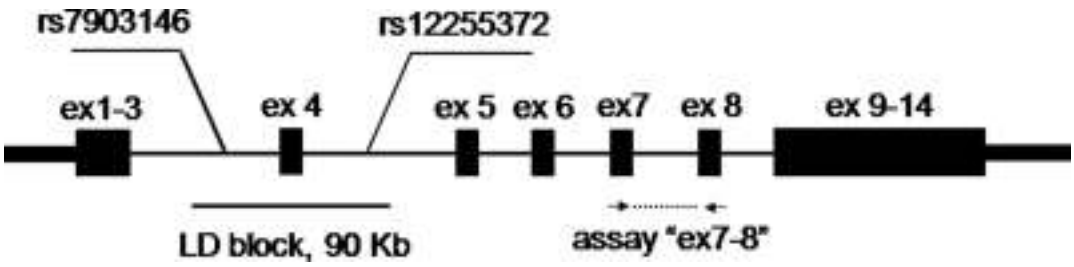
TCF7L2 gene polymorphism in Type 2 Diabetes :

Among the various transcriptional factors that regulate Proglucagon gene expression, Wnt pathway have showed a promising effect in the transcription of Proglucagon gene resulting in GLP-1 release in intestinal cells and also insulin synthesis and release in beta cells of pancreas.

In the homeodomain β cat/ TCF7L2 that mediates Wnt pathway for transcription of proglucagon gene, the transcription factor TCF7L2 exerts important regulatory action in initiating transcription and synthesizing glucagon- like – peptide -1 in the enteroendocrine L cells. The SNP of TCF7L2 gene leads to altered GLP-1 level, reduced postprandial insulin secretion[48] and enhanced rate of hepatic glucose production and shows strong association with T2DM.

Interestingly all T2DM susceptible TCF7L2 SNPs are located within the intron 3 region of TCF7L2 gene . Hence it is most likely that polymorphism affects the Gene expression and not its function (Shu et al. 2007, Liu & Habener 2008). SNP of TCF7L2 is also associated with Gestational diabetes, Colorectal cancer[110].

TCF7L2 gene polymorphism:



The SNP at rs 7903146 is having strong association with T2DM in many population. There is no convincing association was shown between T2DM risk genotypes and the total expression of TCF7L2 or the level of expression of any TCF7L2 splice variant. Various hypothesis identified are

1. A positively significant correlation was found between exon 4 incorporation in pancreatic islets and hemoglobin A1c levels . Though this need further research, it reveals a link between TCF7L2 splicing varients and plasma glucose levels. Notably, the rs7903146 polymorphism is located within intron 4 which is in close proximity to exon 4[68].
2. A nominally significant difference was observed in genotype dependant relative expression of targeted transcripts using assays for exons 14 to 16[68].
3. TCF7L2 gene carring the risk allele T at rs 7903146 shows an E4BP4 binding site and two CDX1 binding sites in the TCF7L2 protein domains that is absent in the common C allele carrying genotype. This hypothesized that E4BP4, CDX1 and some micro RNAs in the intron3 transcribed with host gene or independently in intron 3 of TCF7L2 leads to a TCF7L2 variant that predisposes to T2DM[68].
4. The alternative splice sites in exon 7 and 9 are highly conserved among species. Exon 9 encodes the recognition site for TLE/Groucho domain. Alternative splicing of this domain did not interfere with TCF7L2 action [68].

Study shows that inclusion of exon 4 found to severely impair TCF7L2-dependant promoter activation, even though specific interactions of TCF7L2 binding partners with the exon 4 – encoded part of the protein have not been demonstrated [68]. Whether this is due to un characterized interactions or due to medium or long range protein structural effects remains to be elucidated.

Due to SNP of TCF7L2 gene, a different splice variant of TCF7L2 isoform were synthesized. Such SNPs showed strong association to T2DM.

TCF7L2 an important transcription factor that initiates transcription of promoter region of proglucagon gene in the presence of Wnt ligands that activates Wnt pathway leads to GLP-1 production in the intestine, insulin synthesis and secretion in the β cells of pancreas and suppression of glucagon secretion in the α cells of pancreas.

In T2DM defective insulin synthesis, defective glucose stimulated insulin secretion and increased hepatic glucose production were contributed due to defective GLP-1 secretion. The genotype showing SNP at rs7901436 and rs12255372 for TCF7L2 gene which affects the transcription of GLP-1 production, has increased incidence of T2DM in various population.

As TCF7L2 the transcription factor, has a significant effect in insulin synthesis and secretion through GLP-1 and independently, it is hypothesized that TCF7L2 gene polymorphism at rs7901436 and rs12255372 could be a genetic marker for Type 2 Diabetes.

MATERIALS AND METHODS

The study was performed during the period April 2014 – August 2014 as case-control study. Study population had known Type 2 Diabetic subjects as cases and controls were apparently healthy subjects .

STUDY POPULATION:

CASES GROUP:

This group consisted of 44 Type 2 Diabetic patients who were attending OP in the Department of Diabetology, Govt. Kilpauk Medical College & Hospital(GKMH), Chennai. Type 2 Diabetes was considered to be present if an individual had a history of T2DM or if the fasting blood sugar $\geq$126mg/dl, postprandial blood sugar $\geq$ 200mg/dl and HbA1c $\geq$ 6.5[(22)].

INCLUSION CRITERIA:

Only patients with known Type 2 Diabetes Mellitus were included in this study.

EXCLUSION CRITERIA:

- Patients with Type 1 Diabetes,
- Patients with impaired blood glucose level
- Those with other endocrine disorders.

CONTROLS GROUP:

This group consisted of 44 Controls who were chosen from patients attending general Outpatient Department(OPD) at the GKMH, Chennai. Patients were considered controls if an individual had no history of Type 2 Diabetes or if the fasting blood sugar <100 mg% , Postprandial blood sugar <140mg% and HbA1c <5.6[(22)].

The study was approved by the Institutional Ethical Committee of GKMC, Chennai. After a full explanation of the study a written informed consent was obtained from each participant .

SAMPLE COLLECTION:

Under sterile condition, 5ml of peripheral venous blood as a postprandial sample (2 hours after the first bolus of morning breakfast) was withdrawn using sterile disposable syringes from all the study subjects. Then 2ml of this blood was transferred to EDTA tube for HbA1c estimation which was assayed on the same day itself. Remaining 3 ml of blood was transferred to another EDTA tube and mixed thoroughly.

BUFFY COAT SEPARATION:

The EDTA tube was centrifuged at 2500 rpm for 20 minutes. Buffy coat was extracted carefully and transferred to 2ml eppendorf. From this buffy coat DNA was extracted using Genomic DNA Minispin Prep kit from HELINI. Plasma separated was aliquoted and stored at -20*C for analysis of GLP-1, Insulin, Blood glucose, Urea, Creatinine, Total.Cholestrol and HDL.

BIOCHEMICAL MARKERS:

Glucogon like peptide-1 and Insulin were determined by enzyme linked immunoassay method. Total cholesterol (TC) and High Density Cholestrol(HDL-c) were determined using Kits in Robonic auto analyser at central biochemistry laboratory, GKMH, Chennai. Non-HDL cholesterol was calculated using formula.

DNA MINIPREPARATION KIT: - From Helini Biomolecules, Chennai.

Kit method allows rapid preparation of high quality genomic DNA. This method is suitable for DNA extraction from blood collected in Heparin or EDTA tube or whole blood treated with citrate or buffy coat. The principle is based on exclusive silica based membrane technology in the form of convenient spin column.

PRINCIPLE :

Cells on short incubation with proteinase K in the presence of chaotropic salts, get lysed and immediately inactivates all nucleases. The Purefast purification filter tube prepacked with special glass fibres allows selective binding to cellular nucleic acids .To remove the contaminating cellular components and purify the bound nucleic acids it undergoes a series of rapid 'spin and wash' steps. Finally the nucleic acids are released from glass fibre by low salt elution.

COMPONENTS OF THE KIT:

· Proteinase K

· Lysis buffer

· Wash buffer I (fresh 70% ethanol)

. Wash buffer II (fresh 70% ethanol)

· Isopropranolol

· Elution buffer

· Spin columns with collection tubes.

PROCEDURE for DNA extraction and purification:

BLOOD / BUFFYCOAT:

A nuclease free 1.5 ml microcentrifuge tube is taken and 200µl of blood or buffy coat followed by following steps.

1. 400µl of lysis buffer was added and mixed well immediately by inversion or gentle vortex.
2. 20µl of Proteinase K was added and vortexed for 10 seconds then allowed for incubation at 56*C for 15 minutes.
3. After incubation 200µl of isopropanolol was added and mixed well by inverting several times.
4. The entire sample is pipetted into the PureFast spin column and centrifuged at 12000 rpm for 1min. The flow-through was discarded and placed the column back into the same collection tube.
5. 500µl of Wash buffer I was added to the PureFast spin column and again centrifuged at 12000 rpm for 1min. Then the flow-through was discarded and placed the column back into the same collection tube.
6. 500µl Wash buffer II was added to the PureFast spin column and again centrifuged at 12000 rpm for 1min. Then the flow-through was discarded and placed the column back into the same collection tube.
7. The step with Wash Buffer II was repeated once again.Then the flow-through was discarded and placed the spin column back into the same collection tube and did empty centrifuge at 13000rpm for 2 min. This step is done to avoid residual ethanol.

8. The PureFast spin column was transferred into a fresh 1.5ml microcentrifuge tube. Then 60µl of the prewarmed Elution Buffer was added at the centre of Purefast spin column membrane. Take care not to touch the membrane with the pipette tip.
9. Incubated at room temperature for 2 mins and centrifuged at 13000rpm for 1min.
10. The column was discarded and the purified DNA extracted was stored at -20*C for future use.

IDENTIFICATION:

Extracted DNA was identified by 1.2% agarose gel electrophoresis by comparing with a known molecular weight, 100 bp DNAladder or Lambda EcoRI / Hind III DNA marker as depicted in (Fig15)

CONCENTRATION OF EXTRACTED DNA:

- Concentration of extracted DNA was estimated using UV spectroscopy at 260nm.The adsorbance at 260nm was 0.0066.

Concentration was calculated usingthe formula: 1 OD is equivalent to50µg/ml

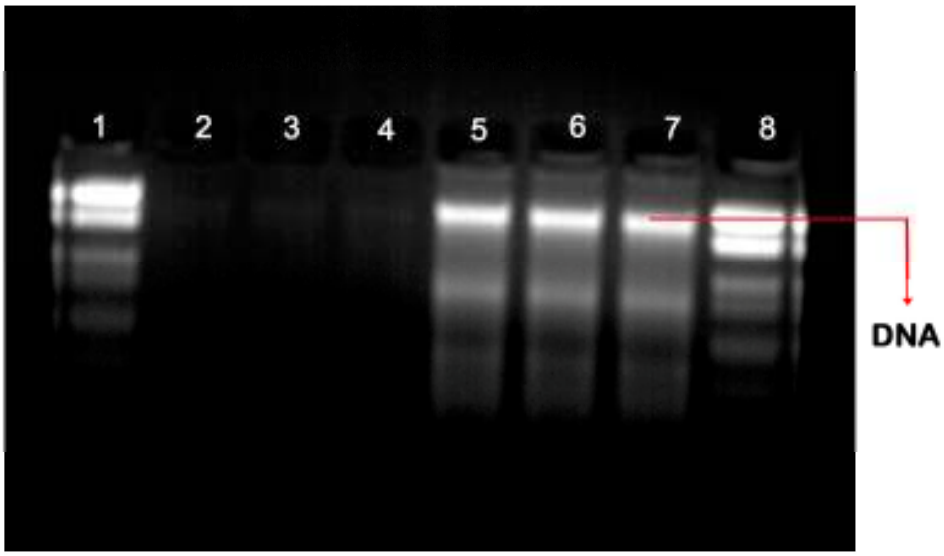
Conc. of DNA = absorbance × 50µg/ml × dilution factor

= 0.0067 × 50 × 60

= 20 ng / µl

• Purity of extracted DNA was assessed by 260/280 ratio and it was found to be > 1.7.

FIG : 15 EXTRACTED DNA



IN TCF7L2 GENE FOR rs7903146:

POLYMERASE CHAIN REACTION:

In TCF7L2 gene for rs7903146, PCR product with 578bp fragment was amplified using the extracted DNA as template and its forward, reverse primers as follows.

Forward primer – 5' – CTGTTTCTTGCTTAGTCACTTTCTG – 3'

Reverse Primer – 5' – CTTTCACTATGTATTGTTGCCAGTC – 3'

PRIMER RECONSTITUTION:

- Primers are supplied in lyophilized form.
- Fresh 1X TAE buffer is used to prepare 100X concentrations .

Forward primer:

Step 1: To prepare forward primer of concentration 100 pmoles/µl solution

- 262 µl of 1X TAE buffer is added to lyophilized primer.

Step 2: From this stock solution, 10X concentration is prepared as working solution for PCR.

- For 200µl of 10X concentration = 10µl primer + 190µl distilled water.

Reverse primer :

Step 1: To prepare forward primer of concentration 100pmoles/µl solution

- 344 µl of 1X TAE buffer is added to lyophilized primer.

Step 2: From this stock solution, 10X concentration is prepared as working solution for PCR.

- For 200µl of 10X concentration = 10µl primer + 190µl distilled water

MASTER MIX:

Heleni 2X PCR master mix in the following composition was used. All basic components needed for PCR were in the Master mix.

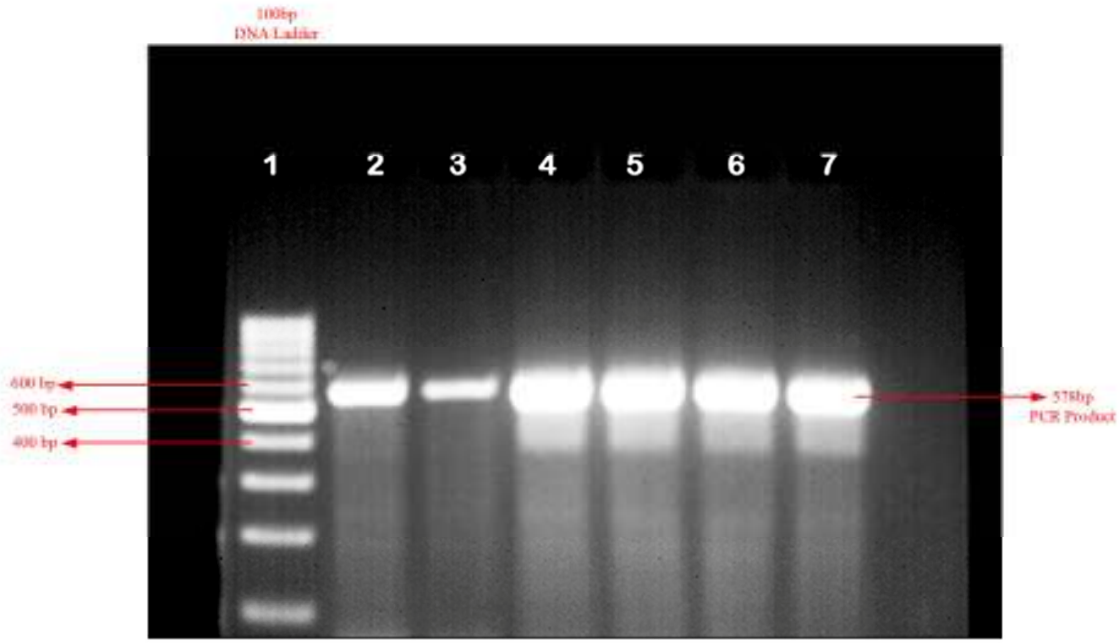
- Reaction buffer consisted of Tris Hcl - 10mM at pH 8.3, KCl - 50mM
- MgCl2 - 2mM acts as catalyst.
- dNTP's were used in a concentration of 200µM each.
- Taq polymerase in a concentration of 1.25 U.
- Primers were used in a concentration of 10pmols and DNA was used in a concentration of 100ng.

PCR was carried out in a reaction volume of 20 µl with the following components:

- PCR master mix – 10 µl
- Forward primer – 2.5 µ l
- Reverse primer – 2.5 µl
- DNA – 5.0µ l
- Total - 20 µl

Amplification was carried out in an CYBERLAB SMART PCR-PRO thermal cycler with the following cycling conditions.

FIG 16 PCR PRODUCT– rs7903146



PCR – RFLP ASSAY done:

The temperature settings for PCR amplification as follows:

- Initial denaturation at 95*C - 5mins
- 34 cycles of
  - Denaturation – 95*C – 30 secs
  - Annealing - 56*C – 30 secs
  - Extension - 72*C – 30 secs
- Final extension at 72*C - 9 min.

The amplified product – amplicons of 578 bp for rs7903146 was identified by 2% agarose gel electrophoresis by comparison with a known 100 bp DNA ladder. (Fig16)

AGAROSE GEL ELECTROPHORESIS:

- PCR product is run on 2% agarose gel in a 30ml agarose cast as follows:

  0.6g of agarose is weighed and dissolved in 30ml of 1X TAE buffer with a pH of 8.2.
  - It is microwaved for 60 secs, cooled and 1.5 µl of ethidium bromide (10mg/ml) is added.
  - It is poured into a cast and allowed to solidify for 45 min before it is kept in the electrophoresis tank.
- 10 µl of PCR product is loaded onto wells and 4 µl of 100bp DNA ladder is loaded onto single well as a marker.
- It is electrophoresed at 8V/cm for 45min and visualized under UV illumination.(fig16)

FIG 18 ARMS – PCR PRODUCT– rs12255372

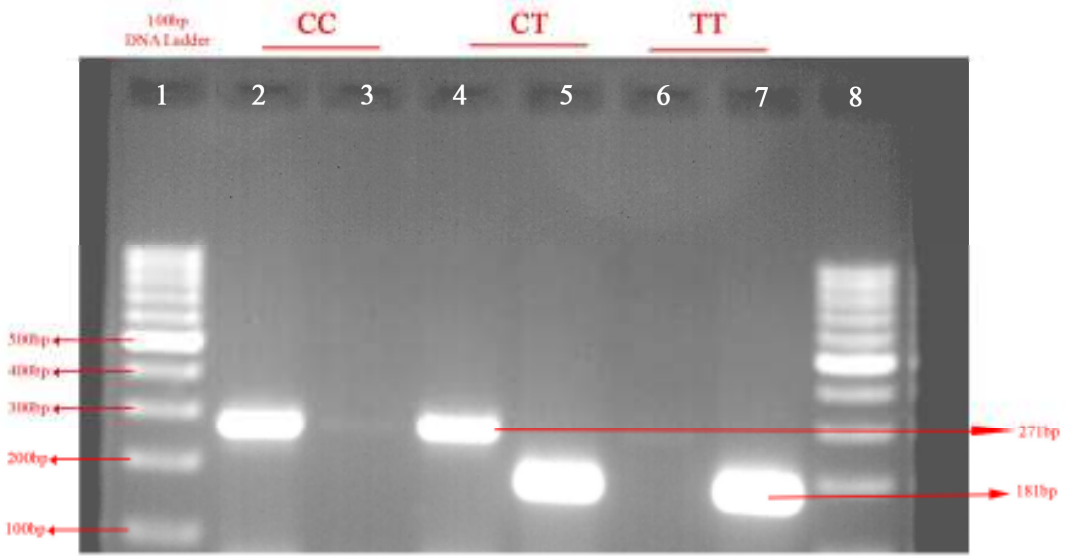
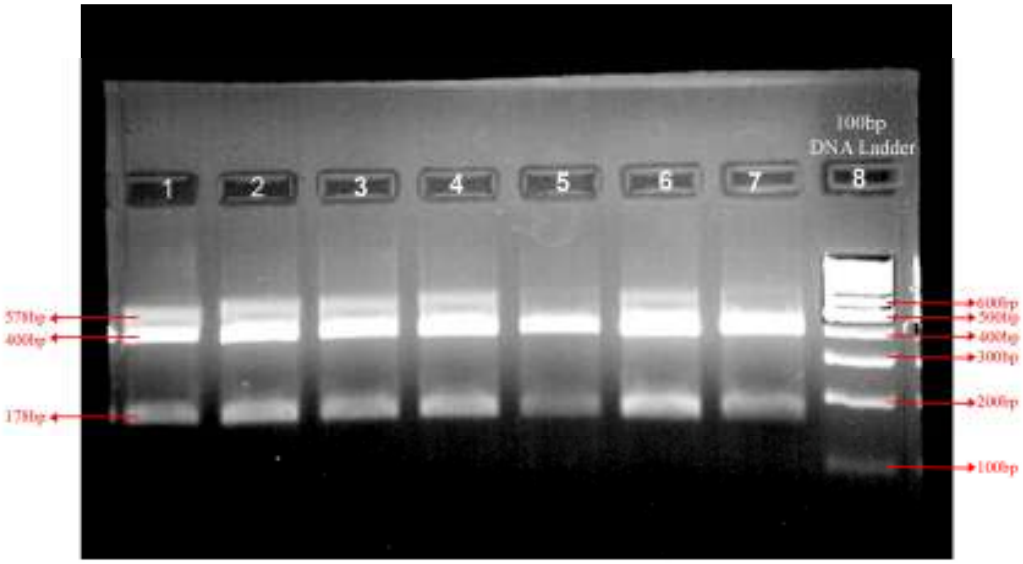


FIG :17 Restriction Digestion Product – rs7903146



RESTRICTION DIGESTION OF PCR PRODUCTS:

TCF7L2 polymorphism at rs7903146 was detected by digestion of the 578 bp PCR amplified product with the Hpy-CH4III restriction enzyme followed by run in 3.0% agarose gel electrophoresis.

Enzyme Taal (HpyCH4III):

- Concentration : 200U / 20 µl.
- 1X Tango buffer : 1 ml

Principle of Hpy-CH4III enzyme digestion:

- T allele does not have the restriction site hence will yield a 578bp fragment
- C allele has the restriction site, hence gets cleaved to give 400bp and 178bp fragments.
- Heterozygous individuals (CT) have 578 bp, 400bp, 178bp fragments.
- Analysis was done using a 100bp DNA ladder.
- The genotype was confirmed by gene sequencing from a random sample.

Procedure for Restriction Digestion:

- 20µL of PCR product is aliquoted in an eppendorf, 3µl of 1X Tango buffer, 6µl of Dist. Water and 10U (1µl) of Hpy-CH4III enzyme is added making a final volume of 30 µl.
- The entire procedure is carried out in ice. The contents are mixed thoroughly.
- The eppendorf is then placed in a 65* C waterbath for 2 hours.

- Restriction digested product is subjected to 3% agarose gel electrophoresis for genotyping.

Procedure for Gel electrophoresis:

- Restriction Digestion product is run on 3.0% agarose gel in a 30mL agarose cast as follows: 0.90g of agarose is weighed and dissolved in 30mL of 1X TAE buffer with a pH of 8.2.
- It is microwaved for 120 secs, cooled and 1.5µL of ethidium bromide (10mg/mL) is added. It is poured into a cast and allowed to solidify for 45 min before it is kept in the electrophoresis tank.
- 20µL of Restriction Digestion product is loaded onto wells and 4µL of 100bp DNA ladder is loaded onto single well as a marker. It is run in electrophoresis tank for 45min and visualized under UV illumination.(Fig.17).

Then the genotypes were identified by comparing the various fragments visualized to 100 bp DNA ladder. The visualized images were Gel documented in LIFE Technologies Gel-doc instrument and stored for future use.

IN TCF7L2 GENE For rs 12255372,

ALLELE SPECIFIC PCR- ARMS ASSAY[147] done.

Primers :

In TCF7L2 gene for rs 12255372, PCR product of G allele with 271 bp fragment was amplified using the extracted DNA as template and its forward, reverse primers as follows.

Forward primer – 5' –GAGGCCTGAGTAATTATCAGAATATGAT**C**– 3'

Reverse Primer – 5' –AGTCATTTGATGATTGTTTTGTTAATGGC– 3'

In TCF7L2 gene for rs 12255372, PCR product of T allele with 181 bp fragment was amplified using the extracted DNA as template and its forward, reverse primers as follows.

Forward primer – 5' –CTGCCCAGGAATATCCAGGCAAGAGTT– 3'

Reverse Primer – 5' –GAGAGAGTGCACTAAAGACGTGGATTC**T**– 3'

The temperature settings for G and T Allele specific PCR amplification which was standardized in our laboratory are as follows:

- Initial denaturation at 95*C - 5mins
- 34 cycles of
    - Denaturation – 95*C – 1 mins
    - Annealing - 64*C – 1 mins
    - Extension - 72*C – 1 mins
- Final extension at 72*C - 5 mins.

The amplified product – amplicons of 271 bp for G allele and 181 bp for T allele, at the rs 12255372 was identified by 2% agarose gel electrophoresis by comparison with a known 100 bp DNA ladder. (fig:18)

AGAROSE GEL ELECTROPHORESIS:

- PCR product is run on 2% agarose gel in a 30 ml agarose cast as follows: 0.6g of agarose is weighed and dissolved in 30ml of TAE buffer with a pH of 8.0.

- It is microwaved for 60 secs, cooled and 1.5 µl of ethidium bromide (10mg/ml) is added. It is poured into a cast and allowed to solidify for 45 min before it is kept in the electrophoresis tank.
- 10 µl of PCR product is loaded onto wells and 4 µl of 100bp DNA ladder is loaded onto a single well as marker.
- Electrophoresis done at 100v for 45min and visualized under UV illumination.

- Then the genotypes were identified by comparing the various fragments visualized to 100 bp DNA ladder. The visualized images were Gel documented in Life sciences technology- gel-doc instrument and stored for future use. The genotype was confirmed by gene sequencing done on a random sample.

The biochemical parameters undertaken for the study were determined using the following methodologies.

- Calibrated using Randox Calibrator Lot no: 2350 – 770UN.
- Randox control lot no: 768UN Level 2 and lot no:501UE Level 3 were used.

Estimation of Plasma Glucose :

Method: Glucose Oxidase peroxidase (GOD / POD)(End point Test)

Kit used : Erba

Principle :

$$\text{Glucose} + O_2 + H_2O \xrightarrow[\text{Peroxidase}]{\text{Glucose Oxidase}} \text{Gluconic acid} + H_2O_2$$

pink coloured

$$H_2O_2 + \text{Phenol} + 4\text{AAP} \longrightarrow \text{Quinoneimine complex} + 2H_2O$$

Intensity of pink coloured Quinoneimine complex is proportional to glucose concentration in the sample . Absorbance was measured at 505 nm.

Reagents composition

Reagent -1 : Enzyme reagent

Glucose oxidase	-	$\geq$ 20000U/L
Peroxidase	-	$\geq$ 2000U/L
Phenol	-	10 mmol/L
Phosphate buffer	-	200 mmol/L
Glucose standard	-	100 mg/dl

Procedure :

To 1000µl of working reagent 10µl of plasma was added and allowed for incubation for 15 mins at 37*C.

Referance range :

Postprandial plasma glucose → 90-140 mg/dl.

Estimation of blood Urea:

Method: Urease - GLDH (kinetic UV test)

Kit used : Accucare

Principle:

Urea is hydrolysed in the presence of water and Urease to produce ammonia and carbondioxide . The ammonia produced combines with α- oxoglutarate and NADH in the presence of glutamate dehydrogenase to yield glutamate and NAD.

$$\text{Urea} + H_2O \xrightarrow{\textit{Urease}} 2NH_4 + CO_2$$

$$NH_4 + \text{NADH} + H^+ + \text{2-oxoglutarate} \xrightarrow{\textit{GLDH}} \text{Glutamate} + NAD^+$$

The initial rate of reduction in absorbance is directly proportional to the urea concentration in the sample. Absorbance is measured at 340 nm.

Reagent composition:

Reagent I : Buffer reagent

Reagent II : Enzyme reagent

Urea standard : 50 mg/dl

Reagent preparation:

Mix 4 parts of Buffer reagent with 1 part of Enzyme reagent and mix gently.

Procedure:

Took 1000µl of reconstituted reagent and added 10µl of plasma and absorbance measured immediately at 340 nm. The rate of reduction after 30 secs and 60 secs is proportional to concentration of Urea.

Referance range: Serum / Plasma Urea → 15 – 39 mg/dl.

Estimation of Creatinine:

Method : Jaffe's method (Picrate method)(Initial rate method)

Kit used: Erba

Principle :

Creatinine reacts with alkaline picrate to produce an orange – yellow colour (Jaffes reaction). Absorbance of the colour is directly proportional to concentration creatinine in the given sample. Absorbance measured at 500nm.

Reagent composition:

Reagent 1 : picric acid reagent

Picric acid - 25.8 mmol/L

Reagent 2 : sodium hydroxide reagent

Sodium hydroxide – 95 mmol/L

Creatinine Standard - 2mg/dl(0.166 mmol/L)

Reagent preparation :

Mix equal volumes of reagent 1 and reagent 2. Wait for 15 mins before use.

Procedure:

Took 1000µl of the reconstituted reagent and added 100µl of the sample and mix and read immediately. Difference between initial absorbance after 20 secs and final absorbance after 80 secs of mixing were measured.

Referance range:

Male : 0.6 – 1.1 mg/dl,

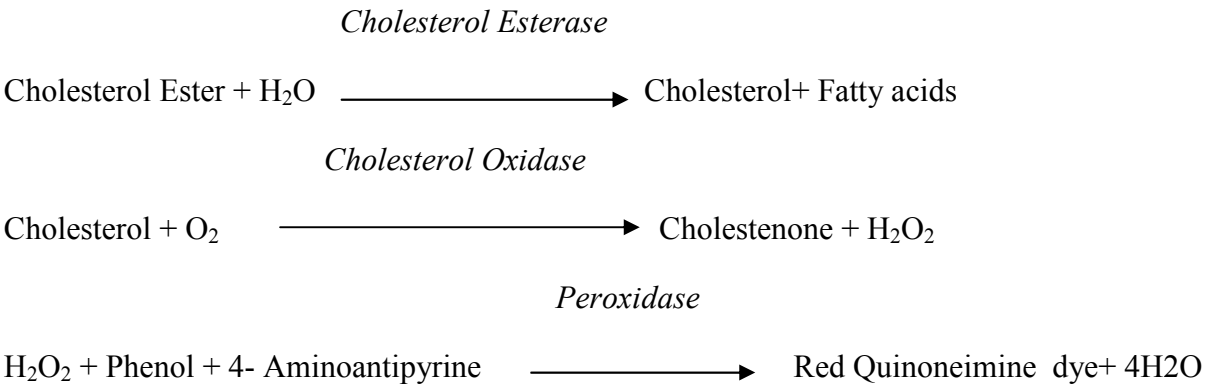
Female : 0.5 – 0.9 mg/dl.

Lipid profile: **Estimation of Cholestrol:**

Method: Cholesterol Esterase – Cholesterol Oxidase CHOD-PAP (End point test)

Kit: Erba

Principle:



The intensity of the red complex (red Quinoneimine) is directly proportional to the concentration of cholesterol in the sample which is measured at 500nm.

Reagent composition :

Buffer (pH – 6.4) - 100mmol/L

Cholestrol Oxidase - > 100 U/L

Cholestrol esterase - > 200 U/L

Peroxidase - >3000U/L

4 – Amino antipyrine - 0.3 mmol/L

Phenol - 5mmol/L

Cholesterol standard - 200mg/dl

Procedure:

To 1000µl of the reconstituted reagent, 10 µl of plasma is added and reading is taken after 5 mins of incubation at 37*C.

Reference Values:

Cholesterol: 150-260 mg /dl.

Estimation of HDL Cholesterol:

Method : Direct, Enzymatic colorimetric

Kit used : Spinreact

Principle:

It is direct determination of serum HDL-cholestrol. The assay takes place in 2 steps:

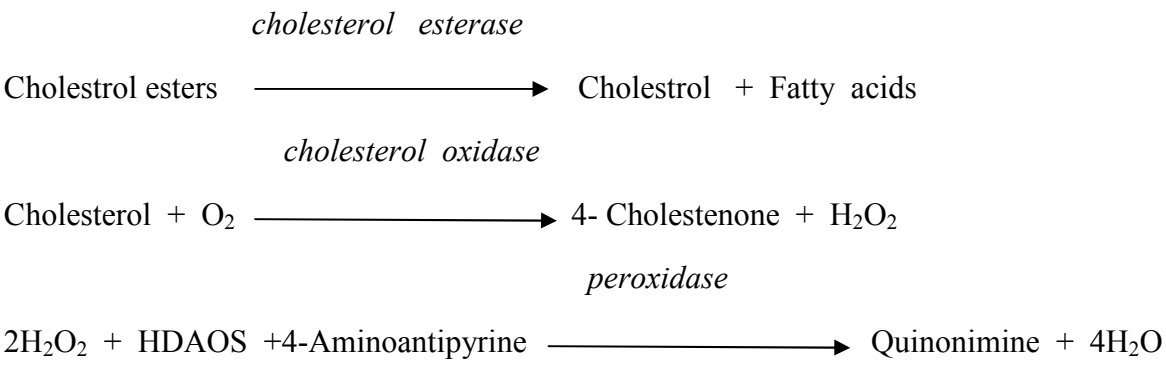
Step 1: Elimination of lipoprotein no-HDL

$$\text{Cholestrol esters} \xrightarrow{\textit{cholesterol esterase}} \text{Cholestrol} + \text{Fatty acids}$$

$$\text{Cholesterol} + O_2 \xrightarrow{\textit{cholesterol oxidase}} \text{4- Cholestenone} + H_2O_2$$

$$2H_2O_2 \xrightarrow{\textit{Catalase}} 2H_2O + O_2$$

Step 2 : Measurement of HDL c



The intensity of the color formed is proportional to HDLc concentration in the sample. Absorbance is measured at 600 nm.

Reagent composition:

Reagent 1:

N-(2-hydroxy-3-sulfopropyl)-3,5-dimethoxyaniline (HDAOS) - 0.7 mM

Cholesterol esterase $\geq$ 800 U/L

Cholesterol oxidase $\geq$ 500 U/L

Catalase $\geq$ 300 U/L

Ascorbic oxidase $\geq$ 3000 U/L

Reagent 2:

4- Aminoantopyrine (4-AP) - 4mM

Peroxidase ≥ 3500U/L

Calibrator :

HDLc - 56.5mg/dl

Procedure :

Reagent 1 and reagent 2 are kept seperatly in auto analyser with the following assay parameters:

Assay type : 2 point

Primary wavelength : 600 nm

Secondary wavelength : 700 nm

R-1 volume : 270,

R-2 volume : 90

Reaction direction : increasing,

Sample volume : 3 µl,

Calibration: straight

Reference Values:

Adult male : 35.3 – 79.5 mg /dl

Adult female : 42.0 – 88.0 mg / dl

Calcualted Non- HDLc:

Non HDLc = Total cholesterol - HDLc

Reference range :(Cardio Metabolic Risk- **CMR**)

- Diabetes with CMR risk factor:

  Non HDLc < 130 mg/dl

- Diabetes without CMR risk factors:

  Non HDLc < 100 mg/dl.

Estimation of Glycated hemoglobin (HbA1C)

Method: High Performance Liquid Chromatography (HPLC)

HbA1C test is done to measure the level of glycated hemoglobin in the blood and reported with eAG- "Average Glucose". This test shows the mean blood glucose level of the patient for the past 2 -3 months**.**

Principle:

Done by Chromatographic assay using BIORAD D10 HPLC instrument, ion exchange column to separate HbA1c molecules from other hemoglobin molecules. The HbA1c is estimated by the ratio of HbA1c peak area to the total Hb peak areas as given in the equation

eAG(mg/dl) = 28.7 x A1C – 46.7

Reference Range : Hemoglobin A1C (%)

ADA criteria - HbA1c

Normal- < 5.6%

Impaired - 5.7 -6.4%

Diabetic - > 6.5%

Estimation of Glucogon like peptide -1:

Method : Competitive Enzyme Immunoassay

Kit : RayBiotech^{R}EIA

Principle:

The microplates are precoated with anti-rabbit secondary antibody. The plate is incubated with anti GLP-1 antibody . During the incubation time both biotinylated GLP-1 peptide and peptide standard or targeted peptide in samples react competitively with the GLP-1 antibody. Bound or Uncompeted biotinylated GLP-1 peptide interacts with streptavidin – horseradish peroxidase (SA-HRP) and catalyzes a color development reaction. Colorimetrically the intensity measured is directly proportional to the amount of biotinylated peptide –SA –HRP complex and inversely proportional to the amount of GLP-1 peptide present in the standard or samples. With known concentration of GLP-1 peptide a standard curve can be established from which the concentration of GLP-1 peptide in the samples are calculated accordingly.

Kit component:

1. GLP-1 Microplate : 96 wells coated with secondary antibody.
2. Anti-GLP-1 polyclonal antibody : 2 vials, 5µl/vial
3. 1x Assay Diluent E: 2 vial, 25 ml (diluents for both standards and samples)

4. Standard GLP-1 Peptide : 2 vials, 10 µl/vial
5. Biotinylated GLP-1 peptide : 2 vials, 20 microlitre/vial
6. Positive control : 1 vial, 100µl.
7. TMB one-step substrate reagent :12 ml of 3,3',5,5'-TetraMethylBenzidine in buffered solution.
8. Wash buffer concentrate (20x): 25 ml
9. Stop solution :8ml of 0.2 M sulfuric acid.
10. HRP – streptavidin concentrate : 600µl, 250x concentrated HRP- conjugated streptavidin

Reagent preparation:

Kit reagents must be kept on ice during preparation.

1. Anti-GLP-1 polyclonal antibody concentrate:

   Centrifuge the vial .Prepared polyclonal antibody concentrate add 50µl of 1x Assay Diluents E and pipette up and down to mix gently.

2. Anti-GLP-1 antibody working solution :

   Polyclonal Antibody concentrate should be diluted 100 folds with 1x Assay Diluent E

3. Biotinylated GLP-1 with final concentration 10pg/ml:

   The vial is centrifuged and add 5µl of biotinylated GLP-1 to 5ml of 1x Assay Diluent E . Pipette up and down to mix gently.

4. Standard preparation :

   - With the following concentrations 6 microtubes are labeled as 1000 pg/ml, 100pg/ml, 1pg/ml, 0.1 pg/ml,0 pg/ml.

- Except for 1000pg/ml tube, add 450 µl of biotinylated GLP-1 solution into each tube.
- Standard-1 , 1000pg/ml GLP-1 stock solution:

  In 1000pg/ml labeled tube, added 8 µl of standard GLP-1 + 792 µl of 10pg/ml biotinylated GLP-1.
- Standard -2, 100pg/ml :

  In 100pg/ml labeled tube having 450 µl of biotinylated GLP-1 added 50 µl of GLP-1 stock solution and mixed thoroughly.
- Standard – 3, 10pg/ml :

  In 10pg/ml labeled tube having 450 µl of biotinylated GLP-1 added 50 µl of standard 2 solution and mixed thoroughly.
- Standard – 4, 1pg/ml:

  In 1pg/ml labeled tube having 450 µl of biotinylated GLP-1 added 50 µl of standard 3 solution and mixed thoroughly.
- Standard – 5, 0.1pg/ml :

  In 0.1pg/ml labeled tube having 450 µl of biotinylated GLP-1 added 50 µl of standard 4 solution and mixed thoroughly.
- Standard – 6, 0pg/ml :

  In 0 pg/ml labeled tube has only 450 µl of biotinylated GLP-1 of 10 pg/ml concentration with 0pg/ml of GLP-1 concentration.

5. 10 fold diluted biotinylated GLP-1 :

   2 µl of biotinylated GLP-1 + 18µl of 1x Assay Diluent E.

6. 2 fold diluted Positive control preparation :

   -Centrifuge the positive control vial -100µl.

-add 101µl of 1X Assay Diluents E and add 2µl of 10 fold diluted biotinylated GLP-1 to the 100µl of positive control.

7. 20X wash buffer concentrate – 20ml:

   Prepared 400ml of 1X wash buffer – 20ml of wash buffer concentrate + 380ml of deionised water .

8. Sample preparation :

   Add 2.5µl of 10 – fold diluted biotinylated GLP-1 to 247.5 µl of sample and mix thoroughly.

9. HRP – streptavidin conjugate concentrate – 250X - 600µl :

   Short spin the vial . Diluted 250 fold with 1X Assay Diluent E.

Procedure:

Keep the prepared reagent in an ice tray.

- Secondary antibody coated GLP-1 microplate with 96 wells brought to room temperature.100µl of anti-GLP-1 antibody added to each well .Incubated at room temperature for 1.5 hrs with gentle shaking of 1-2 cycles /sec.
- After 1.5 hrs of incubation, the solution in wells are discarded and washed 4 times with 1X wash buffer in an automated plate washer. For complete removal of wash liquid , plate is inverted and blooted against filter paper.
- Add 100µl of standard, positive control and sample into appropriate wells.cover the wells and incubate at RT for 5hrs with gentle shaking 1-2 cycles/sec. Discard the solution and wash .

- Prepared streptavidin solution 100µl added to each well and incubate at RT for 45mins with gentle shaking. After 45mins discard the solution and wash.
- Add 100µl of TMB one-step substrate reagent to each well and incubate for 30mins at RT in dark with gentle shaking.
- After 30mins 50µl of stop solution added to each well and absorbance measured at 450nm immediately in Elisa reader.

Sensitivity :

The minimal concentration is 1.17pg/ml.

Specificity :

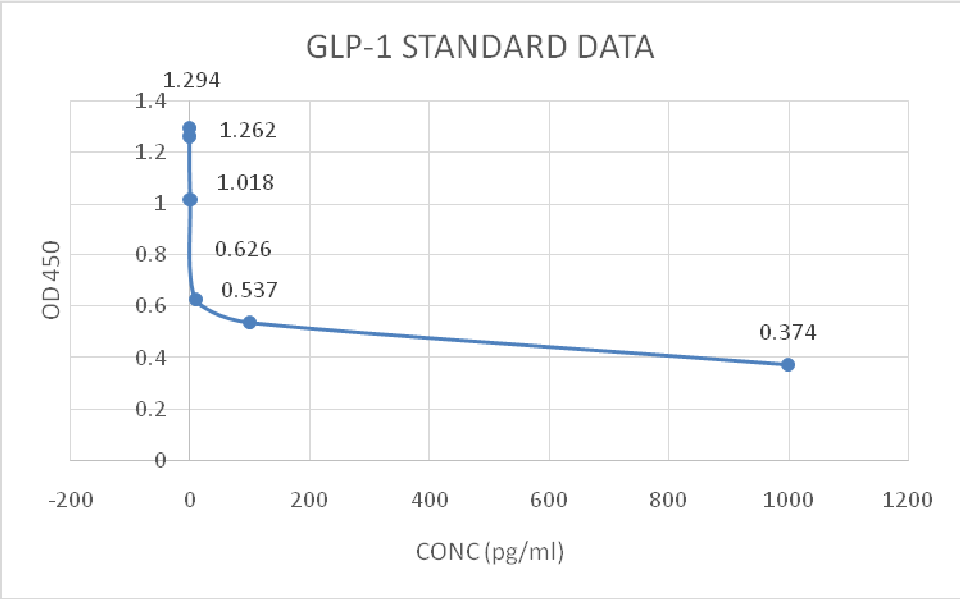
This kit has no cross reactivity with any of cytokines tested – Ghrelin , angiotensin II,

Detection range :

0.1-1000 pg/ml

STANDARD DATA

Standards	**CONC (pg/ml)**	**OD 450**
S0	1000	0.374
S1	100	0.537
S2	10	0.626
S3	1	1.018
S4	0.1	1.294
S5	0	1.262


GLP-1 STANDARD DATA
1.294
1.262
1.018
0.626
0.537
0.374
OD 450
CONC (pg/ml)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
-200
0
200
400
600
800
1000
1200

Estimation of insulin level:

Method: Enzyme linked immunoassay

Kit: DRG diagnostics

Principle:

This is a solid phase enzyme – linked immunosorbent assay(ELISA) based on sandwich principle. The monoclonal antibody coated on microtitre wells bind to unique antigenic site on the insulin molecule. On incubation endogenous insulin that bound to monoclonal antibody, reacts and binds to enzyme conjucate - anti-insulin antibody conjugated to biotin. After unbound conjugates are washed, Enzyme complex - Streptavidin –HRP Complex binds to biotin anti insulin antibody. The amount of bound HRP complex is proportional to concentration of insulin in the sample. Having added the substrate solution, the intensity of colour developed is proportional to the concentration of Insulin in the patient sample.

REAGENTS :

- Microtitre wells- 96 wells ,coated with monoclonal anti-insulin antibody.
- Standards – 0, 6.25,12.5, 25, 50, 100 μIU/ml
- Enzyme conjugate – Monoclonal anti-insulin conjugated to biotin.
- Enzyme complex- Streptavidin – HRP-complex
- Substrate solution – Tetramethylbenzidium (TMB)
- Stop solution - 0.5M sulphuric acid
- Wash Buffer Concentrate: 40X

Wash buffer concentrate, 30ml is mixed with 1170ml of deionized water to prepare wash buffer of final volume 1200 ml. Keep it at the room temperature.

ASSAY PROCEDURE

Bring all reagents to room temperature, before assay. Proper mixing of reagent is done before assay.

1. Desired wells are secured into the holder.
2. Using new disposable tips, 25 µl of standards, control and patient's samples are Pipetted into desired wells.
3. Insulin Enzyme Conjugate of 25µl is added into all wells.
4. Good mixing is done for about 10 sec by gentle shaking.
5. For about 30 minutes incubation done at room temperature.
6. Using automated plate washer washing done . 400 µl of wash buffer is used to Wash wells three times . Blot with absorbent paper.
7. HRP enzyme complex 50µl added to each well.
8. Incubated for 30 mins at RT.
9. Using automated plate washer washing done . 400 µl of wash buffer is used to Wash wells three times. Blot with absorbent paper.
10. TMB substrate of about 50 µl are dispensed to all wells.
11. Incubate for 15 minutes at room temperature.
12. 50µl of stop solution is dispensed to all wells. Gentle Shaking to mix the content in the solution.

13. By ELISA Reader at 450 nm optical density absorbance is read within 10 minutes after adding the stop solution.

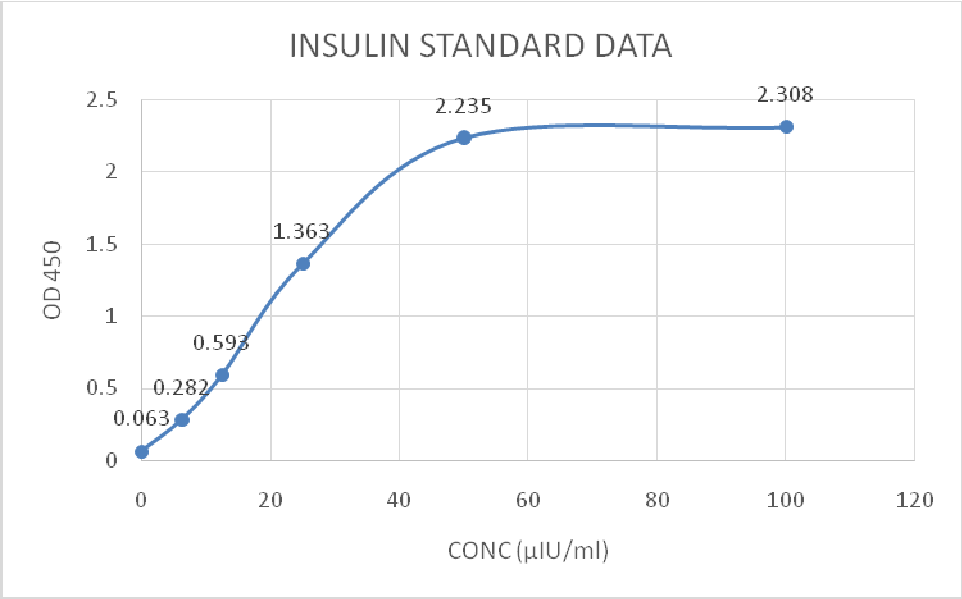
CALCULATION OF RESULTS:

The standard curve is constructed as follows:

1. A standard curve is constructed, by plotting insulin standards absorbance value along the vertical axis versus the insulin standard concentrations in µIU/ml along horizontal axis .
2. Unknown sample concentration is read from the standard curve. Record the value for each control or unknown sample.
3. Value above the highest point of the standard are retested after diluted or reported as > 100µl /ml.

STANDARD DATA

Standards	**CONC (µIU/ml)**	**OD 450**
S0	0.000	0.063
S1	6.250	0.282
S2	12.50	0.593
S3	25.00	1.363
S4	50.00	2.235
S5	100.00	2.308


INSULIN STANDARD DATA
OD 450
2.5
2
1.5
1
0.5
0
0.063
0.282
0.593
1.363
2.235
2.308
0
20
40
60
80
100
120
CONC (µIU/ml)

PERFORMANCE CHARACTERISTICS:

Assay dynamic range -1.76 to 100µIU/ml.

Sensitivity:

This is measured by calculating the mean plus 2 Std Deviation of the standard zero point tested twenty times in the same run and was found as 1.76µIU/ml.

Specificity:

The antibodies used in the kit cross react with porcine insulin and bovine insulin (>100%) but did not cross react with human proinsulin .

RESULTS

In Chennai suburban population, 44 Type 2 Diabetic cases and 44 apparently healthy controls were analysed for genotype distribution of TCF7L2 gene. Association of each genotype with its clinical and biochemical parameters were studied.

In Master table, the characteristics of Type 2 Diabetes Mellitus patients [1 to 44] and Control group [45 to 88], along with their TCF7L2 genotyping and biochemical parameters are given.

Statistical analysis:

- For standard statistical analysis of the data's, Statistical products and service solutions (SPSS) package were used.
- The biochemical parameters between type 2 diabetic cases and healthy controls were tested by using students t test .
- The frequency of Genotype distribution between cases and controls were compared by using Chi-square (χ^2) test.
- In logistic regression analysis, Odds ratio with two tailed p values and 95% confidence intervals (CI) were calculated.
- Level of significance for p-value was set at point < 0.05. If $p < 0.001$ shows strongly significant.
- Hardy- Weinberg law was followed and the frequency of genotype were tested for Hardy – Weinberg equilibrium.

Table -1: Comparison of age between cases and controls.

Variables	Group	N	Mean	Std. Dev	t-Value	P-Value
Age (years)	Cases	44	49.75	8.535	0.214	0.921
	Controls	44	45.23	8.364		

Table 1 : The patients were with a mean age of 49.75 + 8.535 years, while the controls were with a mean age of 45.23± 8.364 years. No Significant differences were observed between the mean ages of patients and controls (p=0.921).

Table – 2: Gender distribution among cases and controls:

Gender	Group						**Chi-Square Test**	P-Value
	Cases		Controls		Total			
	N	%	N	%	N	%		
Male	20	45.5	20	45.5	40	45.5	1	0.585
Female	24	54.5	24	54.5	48	54.5		

Table 2 shows among diabetic cases, 20 were males (45.5%) and 24 were females (54.5%). Among controls, 20 were males (45.5%) and 24 were females (54.5%). No significant difference in gender distribution between cases and controls (p = 0.585) was observed.

Table -1: Comparison of age between cases and controls.

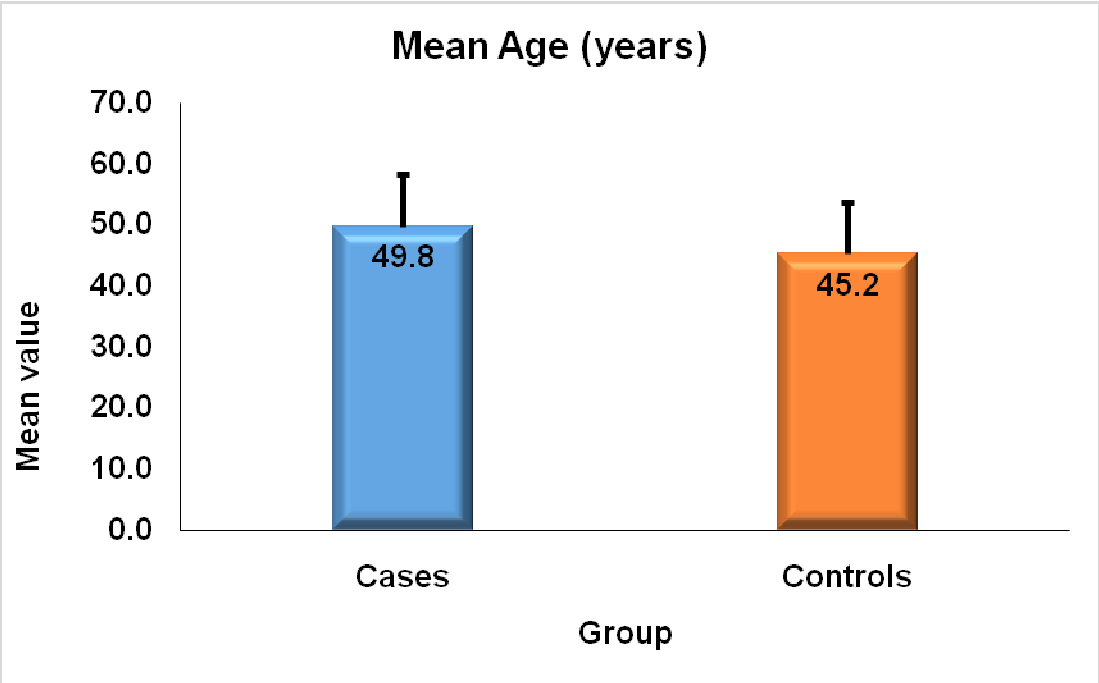


Table – 2: Gender distribution among cases and controls:

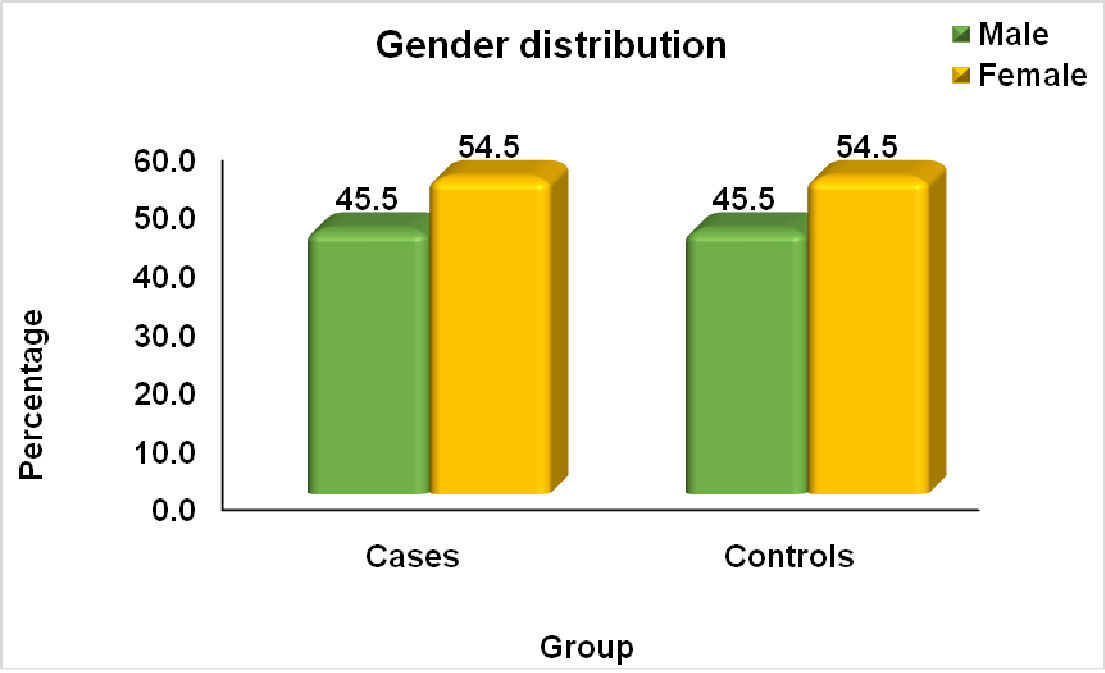


Table 3:Comparison of BMI and Waist circumference between cases and controls:

S.No	Variables	Group	N	Mean	Std. Dev	t-Value	P-Value
i.	BMI	Cases	44	25.52	4.717	1.764	0.081
		Controls	44	27.06	3.348		
ii.	Waist (cm)	Cases	44	103.4	15.57	4.186	<0.001
		Controls	44	94.5	8.15		

i. Mean BMI of cases and controls were 25.52 $\pm$ 4.717 and 27.06 $\pm$ 3.348 respectively, (p = 0.081).

ii. Mean waist circumference of cases and controls were 103.4 $\pm$ 15.57 and 94.5 $\pm$ 8.15 cms. The mean value of cases are higher than controls and the difference is significant with p value = <0.001.

Table 3:Comparison of BMI circumference between cases and controls:

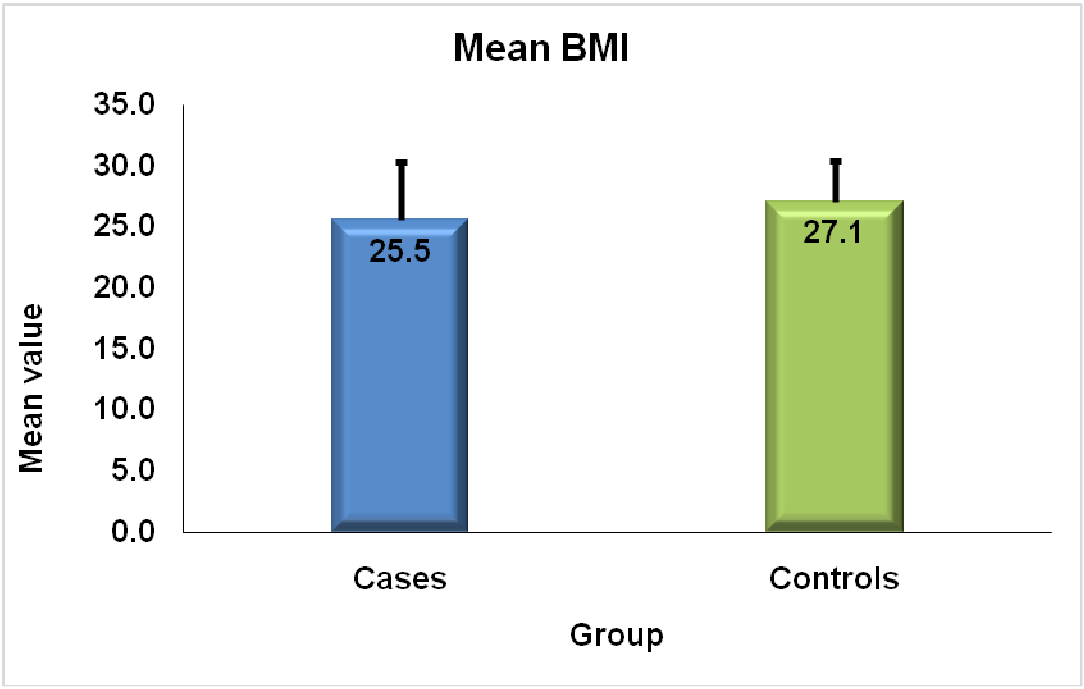


Table 3:Comparison of Waist circumference between cases and controls:

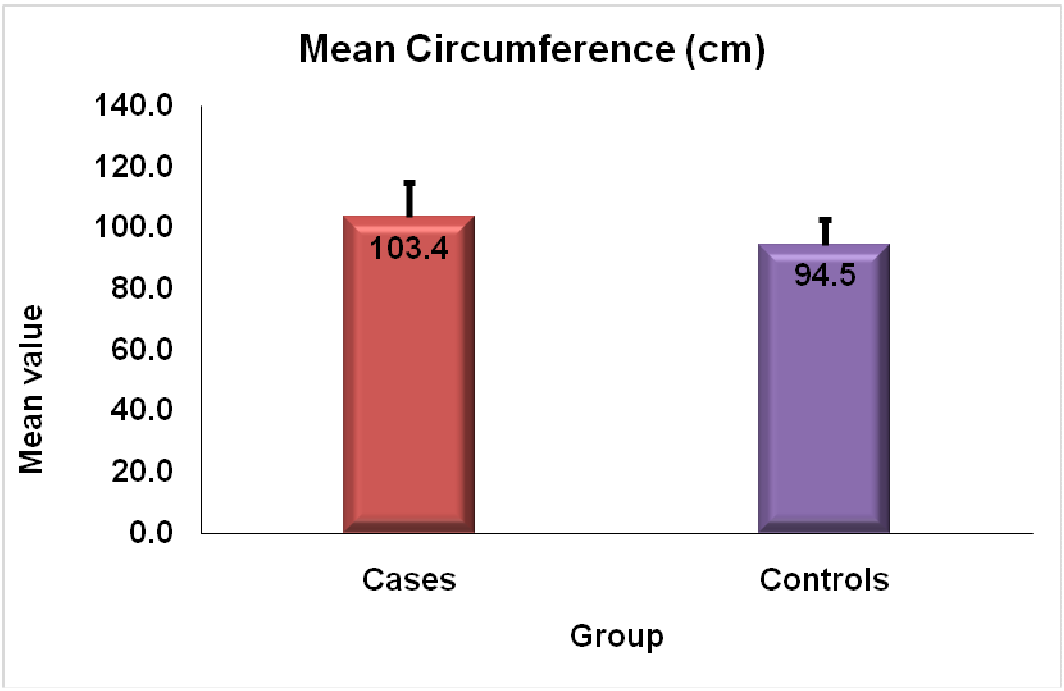


Table 4: Comparison of Urea and Creatinine between cases and controls:

S.No	Variables	Group	N	Mean	Std. Dev	t-Value	P-Value
i.	Urea (mg/dl)	Cases	44	24.05	4.617	1.321	0.190
		Controls	44	25.54	5.929		
ii.	Creatinine (mg/dl)	Cases	44	0.862	0.150	3.179	0.002
		Controls	44	0.965	0.156		

i. The mean values of blood Urea in cases and controls were 24.05 $\pm$ 4.617 and 25.54 $\pm$ 5.929 mg/dl respectively with no difference in Urea levels between the study groups $p = 0.190$.

ii. The mean value of creatinine in cases were 0.862 $\pm$ 0.150 and controls were 0.965 $\pm$ 0.156 mg/dl and shows difference in creatinine level between cases and controls with a p value = 0.002.

Table 4: Comparison of Urea between cases and controls:

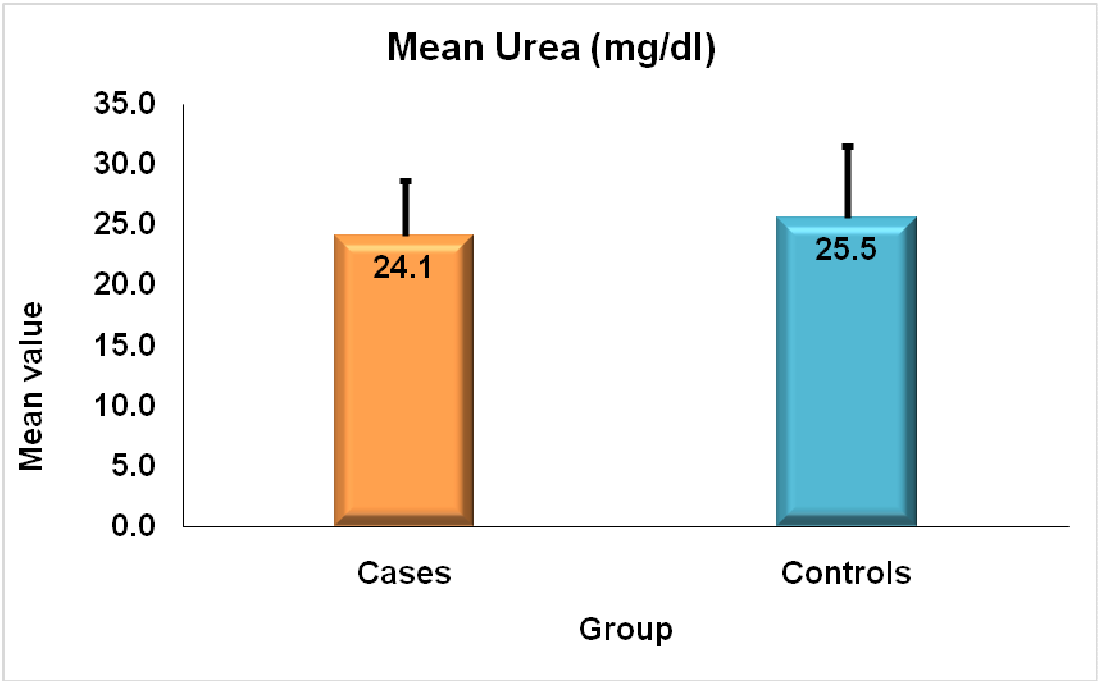


Table 4: Comparison of Creatinine between cases and controls:

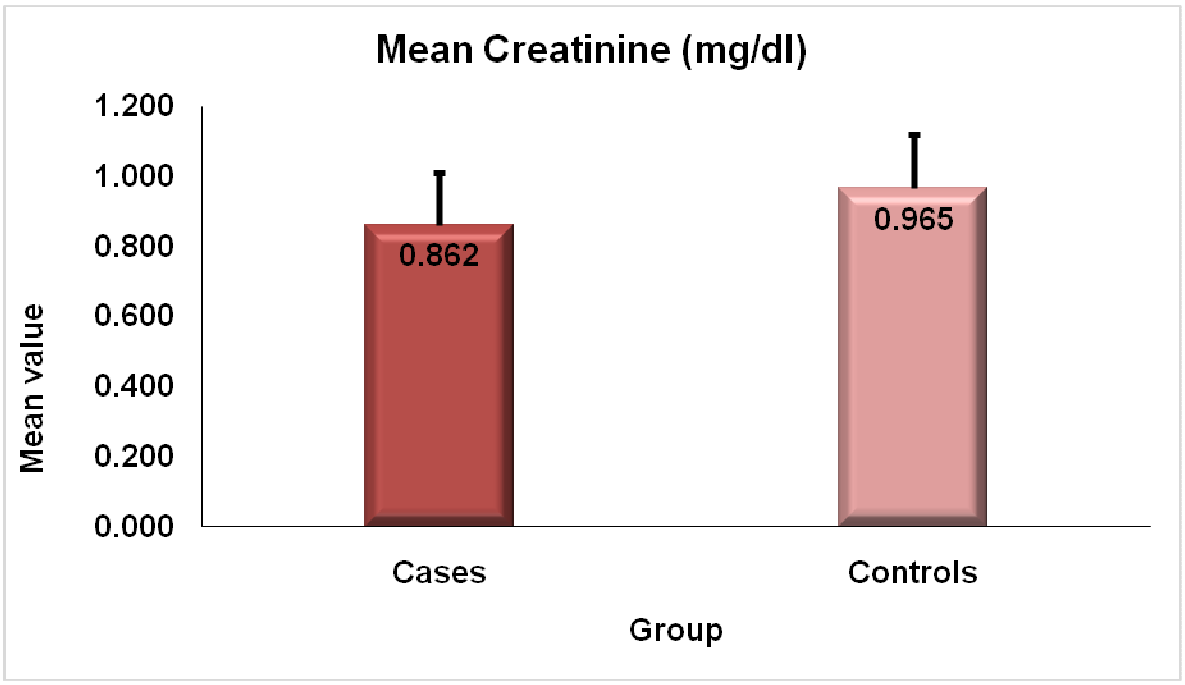


Table 5: Comparison of Glucose , HbA1c , Insulin and GLP-1 between cases and controls:

S.No	Variables	Group	N	Mean	Std. Dev	t-Value	P-Value
i.	Glucose (mg/dl)	Cases	44	244.8	85.30	10.211	<0.001
		Controls	44	108.6	23.50		
ii.	HbA1C	Cases	44	8.416	1.830	11.363	<0.001
		Controls	44	5.218	0.371		
iii.	Insulin (µIU/ml)	Cases	44	276.0	332.4	0.993	0.323
		Controls	44	211.2	277.6		
iv.	GLP-1 (pg/ml)	Cases	44	0.101	2.353	6.045	<0.001
		Controls	44	3.588	3.294		

i. The mean 2 hours postprandial plasma glucose values in cases and controls are 244.8 mg/dl $\pm$ 85.30 and 108.6 mg/dl $\pm$ 23.50 respectively ($p < 0.001$) .

ii. The mean HbA1C values in cases and controls are 8.416 ± 1.830 and 5.218 ± 0.371 respectively ($p < 0.001$).

iii. The mean value of insulin in cases and controls are 276.0 ± 332.4 and 211.2 ± 277.6 µIU/ml respectively($p = 0.323$).

iv. The mean value of GLP-1 measured in 2 hours post prandial sample in cases and controls are 0.101 ± 2.353 and 3.588 ± 3.294 respectively ($p< 0.001$) .

Table 5:Comparison of Glucose between cases and controls:

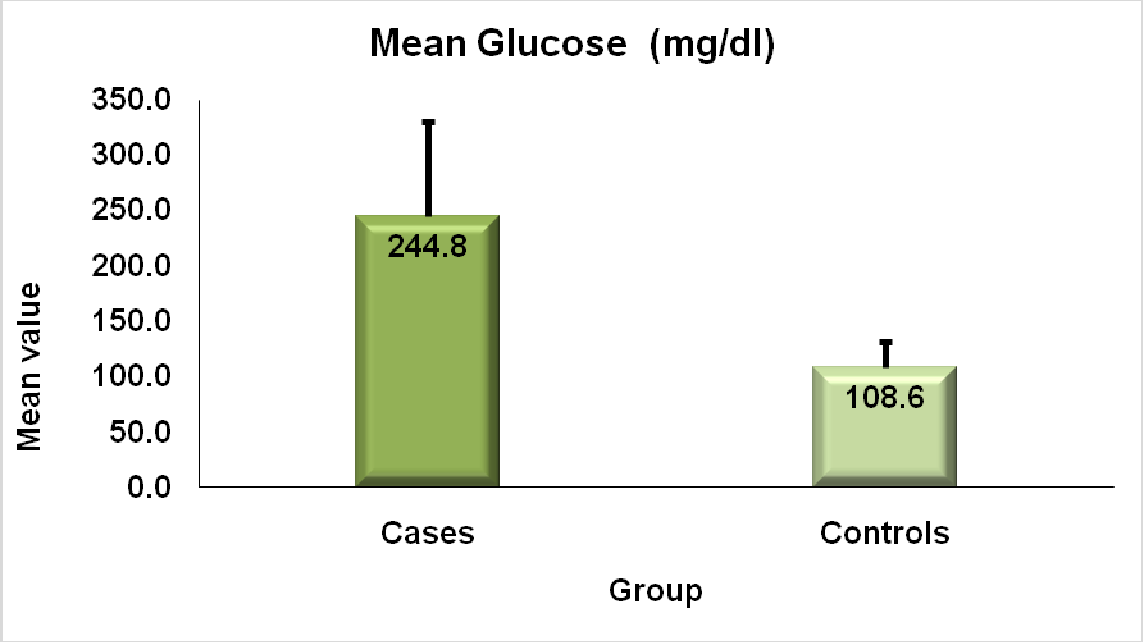


Table 5:Comparison of HbA1c between cases and controls:

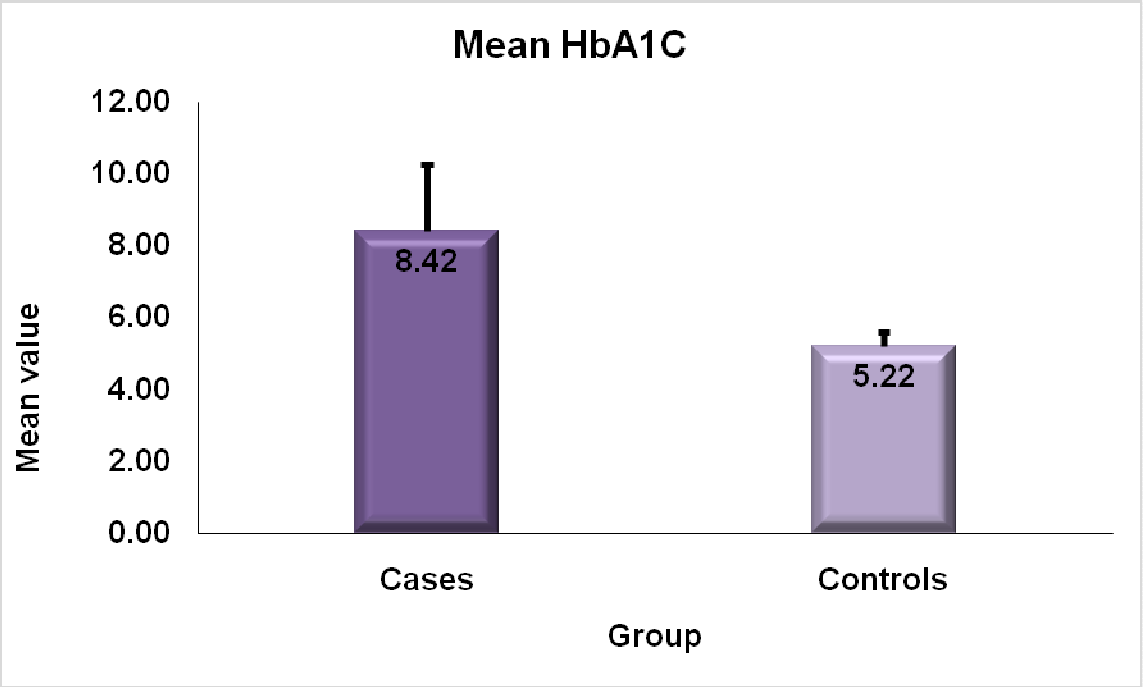


Table 5:Comparison of Insulin between cases and controls:

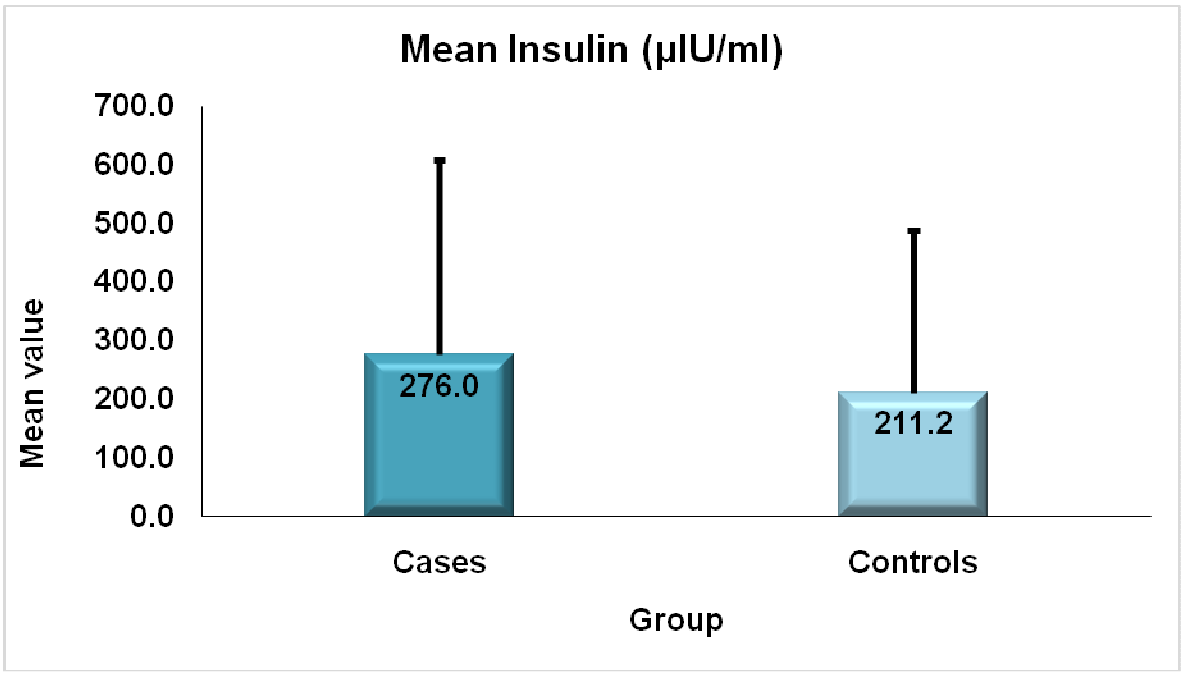


Table 5: Comparison of GLP-1 between cases and controls:

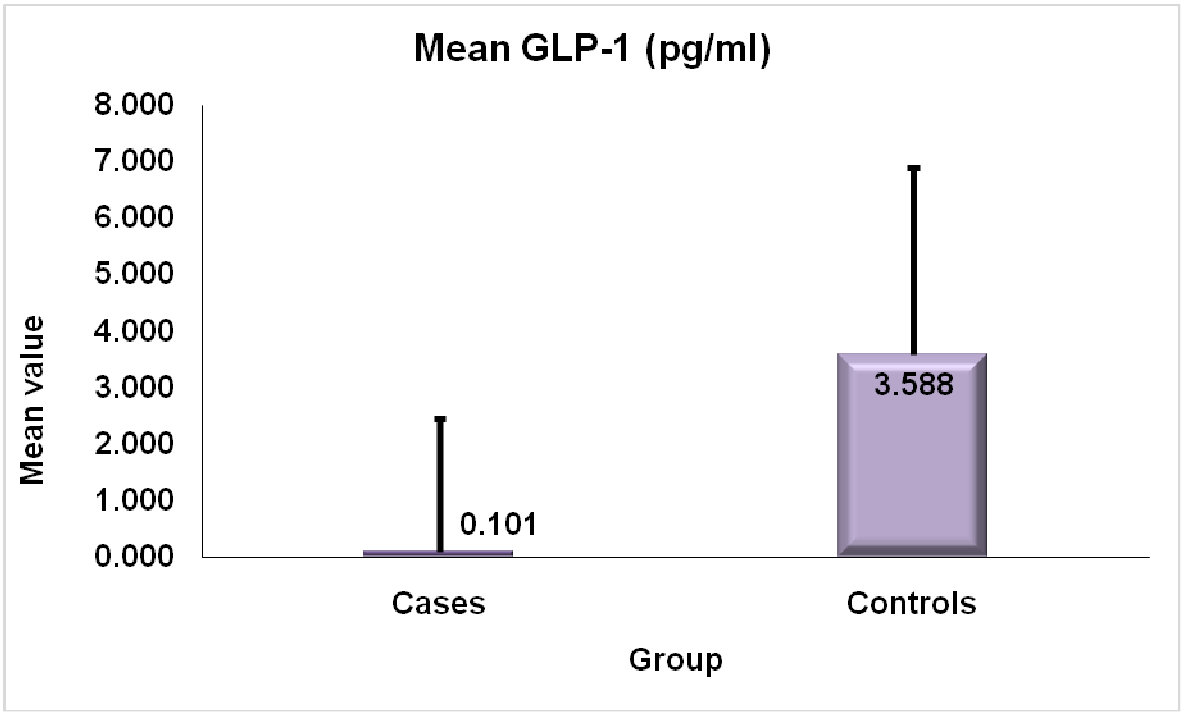


Table 6: Comparing mean values of postprandial lipid profile between cases and controls:

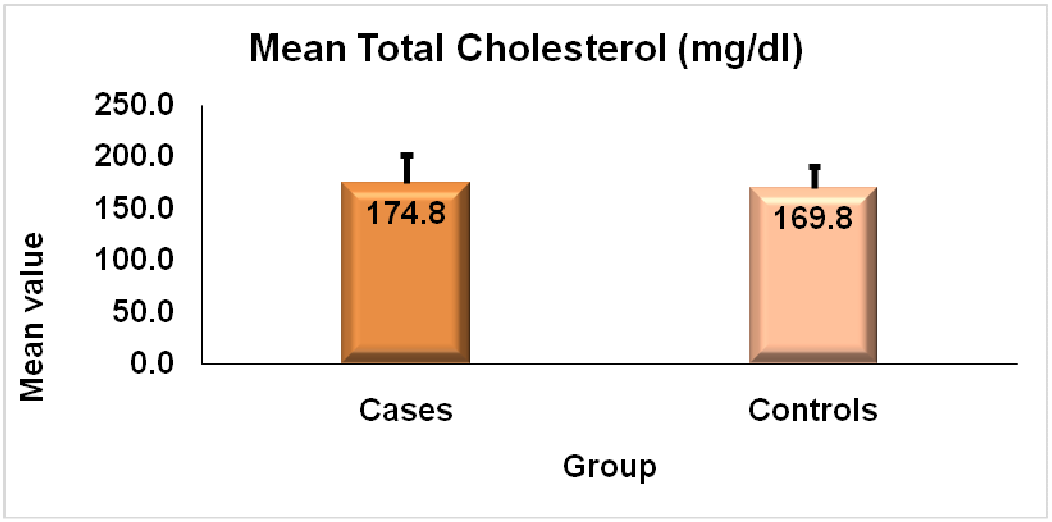
S.No	Variables	Group	N	Mean	Std. Dev	t-Value	P-Value
i.	T.Cholesterol (mg/dl)	Cases	44	174.8	27.163	0.990	0.325
		Controls	44	169.8	20.564		
ii.	HDL-c (mg/dl)	Cases	44	43.78	8.770	0.510	0.611
		Controls	44	44.67	7.472		
iii.	NON-HDL	Cases	44	131.1	25.547	1.220	0.226
		Controls	44	125.1	20.038		

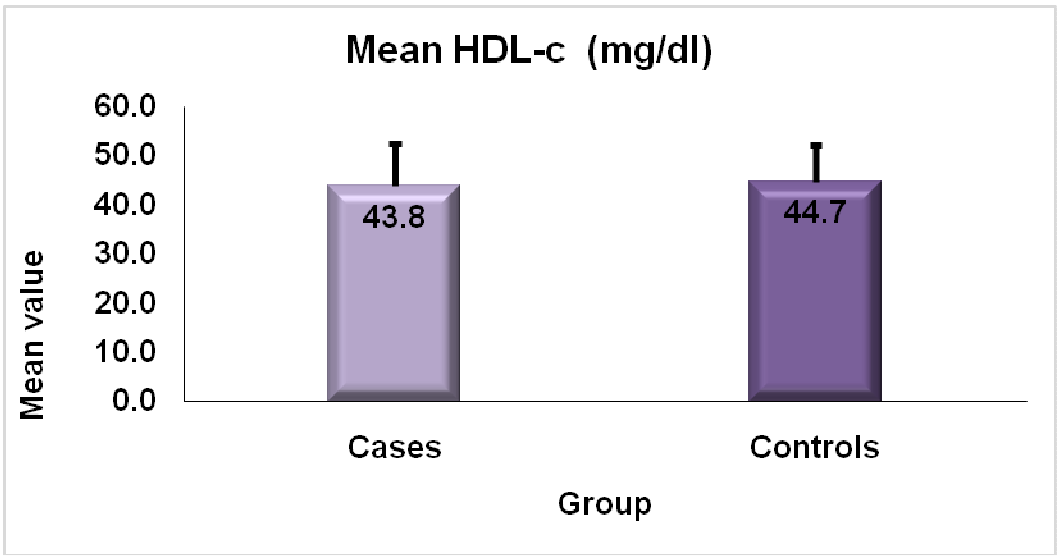
i. The mean T. Cholesterol levels in cases are 174.8 + 27.163 and controls are 169.8 + 20.564 mg/dl ($p = 0.990$).

ii. The mean HDL-c level in cases are 43.78 + 8.770 mg/dl and in controls are 44.67 + 7.472 mg/dl ($p = 0.510$).

iii. The mean Non-HDL –c levels in cases and controls are 131.1 + 25.547 mg/dl and 125.1 + 20.038 mg/dl respectively ($p = 1.220$).

Table 6: Comparing mean values of postprandial lipid profile in cases and controls:





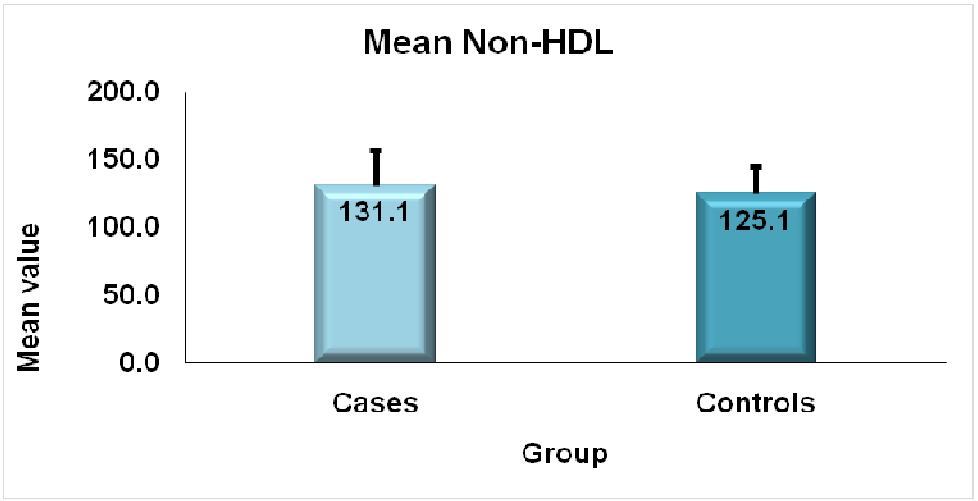


Table -7 : Hardy-Weinberg equilibrium for Gene-1: rs7903146 (cases and controls)

Genotypes	Observed #	Expected #	$\chi 2$	*P* value
Homozygote reference:	59	61.4	3.4249	0.064
Heterozygote:	29	24.2		
Homozygote variant:	0	2.4		

- In the study population , the genotype frequency are CC – 59 (67%) , CT -29 (33%) , TT -0 (0%) .
- By Chi-square test , $\chi^2 = 3.4249$ and p value = 0.064 .
- The genotype distribution in cases and controls obeys Hardy – Weinberg law and is in equilibrium.

Table 8: Genotype distribution of TCF7L2 gene at rs7903146 between diabetic cases and controls :

Gene-1: rs7903146	Group						Pearson Chi-Square test	Odds ratio 95% CI	P value
	Cases		Controls		Total				
	N	%	N	%	N	%			
CC	17	38.6	42	95.5	59	67.0	32.145	0.33 [0.07 -1.56]	<0.001
CT	27	61.4	2	4.5	29	33.0			

Frequency of genotype distribution between cases and controls are as follows :

- CC genotype has a prevalence of 38.6% in cases as compared to 95.5% in controls.
- CT genotype shows 61.4% prevalence in cases and 4.5% in controls.
- TT genotype frequency was not detected in the study population.
- $\chi^2 = 32.145$, $p<0.001$. OR 0.33; 95% CI (0.07-1.56).

Table 8: Genotype distribution of TCF7L2 gene at rs7903146 between diabetic cases and controls :

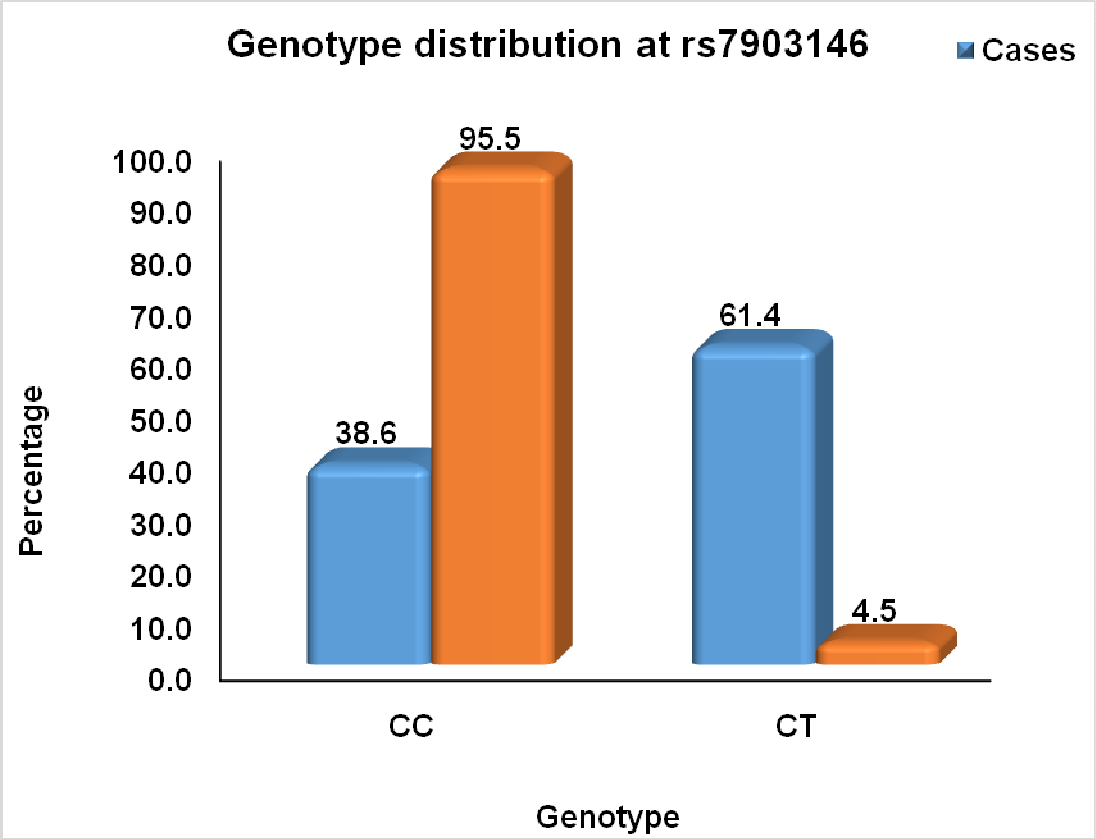


Table 9: Allele distribution of TCF7L2 gene at rs7903146 between diabetic cases and controls:

Gene-1: rs7903146	Group						Chi-Square Test	Odds ratio 95% CI	P-Value
	Cases		Controls		Total				
	N	%	N	%	N	%			
C	61	69.3	86	97.7	147	83.5	25.8	0.19 (0.04-0.83)	<0.001
T	27	30.7	2	2.3	29	16.5			

Frequency of allele distribution between cases and controls are as follows :

- C allele frequency in controls are (97.7%) as compared to cases (69.3%).
- Prevalence of T allele in cases is (30.7%) and in controls is (4.5%).
- $\chi^2 = 25.8$, $p<0.001$, OR 0.19 ;95% CI (0.04-0.83).

Table 9: Allele distribution of TCF7L2 gene at rs7903146 between diabetic cases and controls:

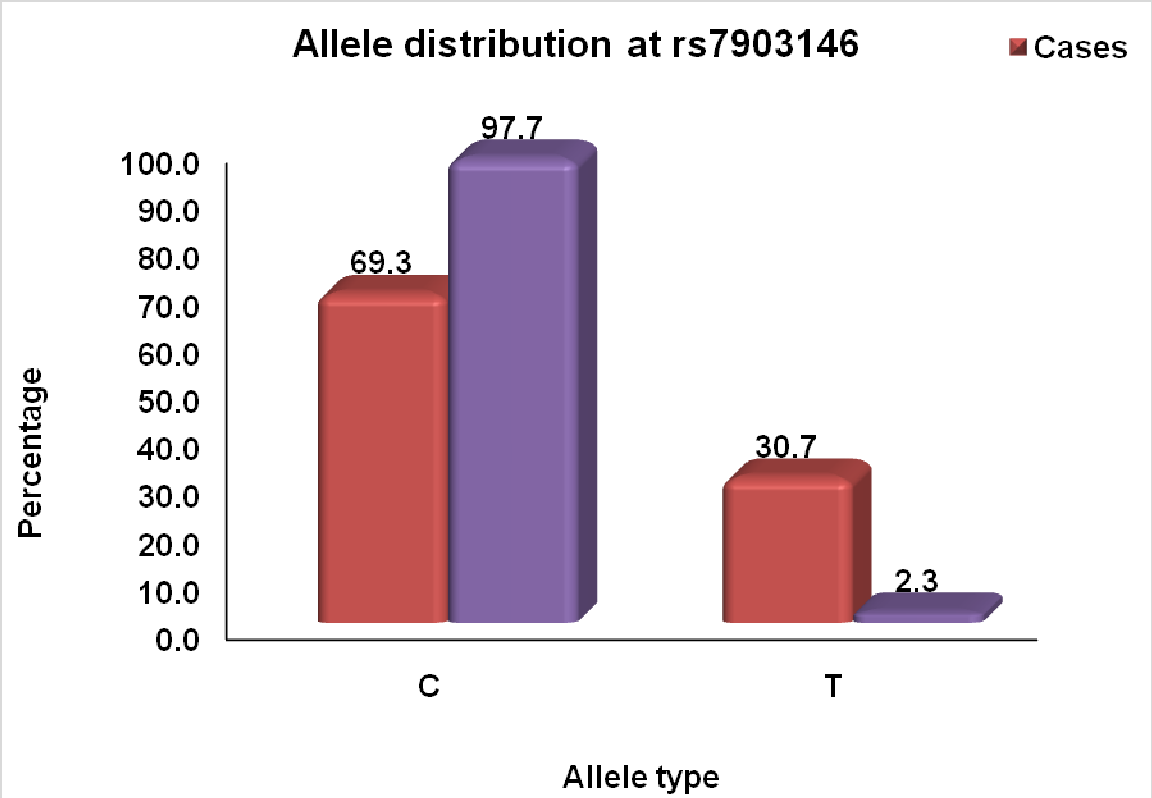


Table 10: Hardy-Weinberg equilibrium for Gene-2: rs12255372 (cases and controls).

Genotypes	Observed #	Expected #	$\chi 2$	P value
Homozygote reference:	56	56.5	0.1032	0.748
Heterozygote:	29	28.0		
Homozygote variant:	3	3.5		

- In the study population , the genotype frequencies are GG – 56 (63.6%) , GT -29 (33%) and TT -3 (3.4%).
- By Chi-square test, $\chi^2 = 0.1032$ and p value = 0.748.
- The genotype distribution in cases and controls obeys Hardy – Weinberg law and is in equilibrium.

Table11: Genotype distribution of TCF7L2 gene at rs12255372 between cases and control:

Gene-2: rs12255372	Group N %		Odds ratio 95% CI	Chi-Square Test	P-Value
	Cases	Controls			
GG	24 (54.5%)	32 (72.7%)	reference	4.299	0.112
GT	19 (43.2%)	10 (22.7%)	2.53 [1.01-6.42]		
TT	1 (2.3%)	2 (4.5%)	0.67 [0.06-7.79]		

Frequency of genotype distribution between cases and controls are as follows :

- GG genotype has a prevalence of 54.5% in Cases as compared to 72.7% in controls.
- GT genotype shows 43.2% prevalence in cases and 22.7% in controls OR-2.53; 95%CI [1.01-6.42].
- TT genotype frequency is 2.3% in cases as compared to 4.5% in controls, OR-0.67; 95%CI [0.06-7.79].
- $\chi^2 = 4.299$, p=0.112.

Table11: Genotype distribution of TCF7L2 gene at rs12255372 between cases and control:

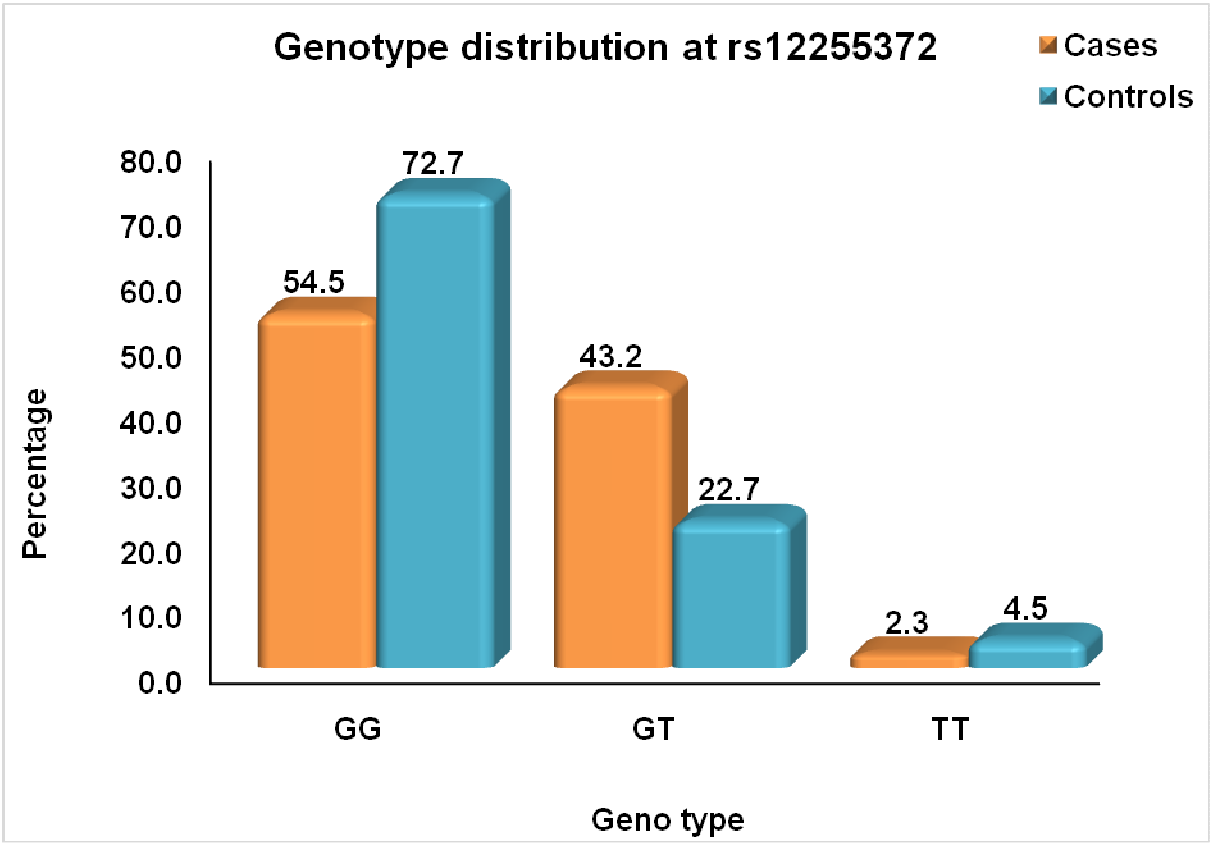


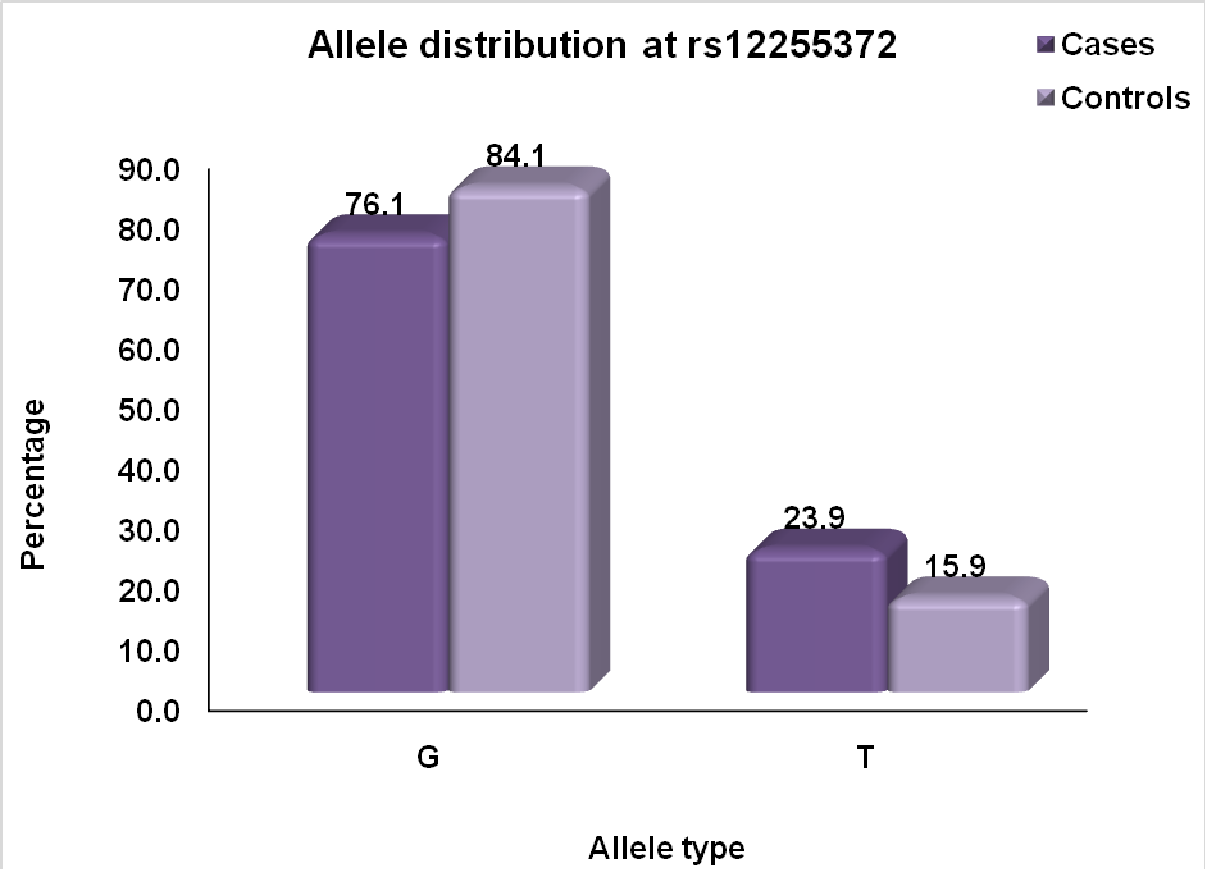
Table12 : Allele distribution of TCF7L2 gene at rs12255372 between cases and control:

Gene-2: rs12255372	Group N %		Odds ratio 95% CI	Chi-Square Test	P-Value
	cases	controls			
G	67 (76.1%)	74 (84.1%)	1.66 [0.78-3.53]	1.748	0.093
T	21 (23.9%)	14 (15.9%)			

Frequency of allele distribution between cases and controls are as follows :

- G allele frequency in controls is (84.1%) as compared to cases (76.1%).
- Prevalence of T allele in cases (23.9%) and in controls (15.9%) .
- χ^2 = 1.748, p=0.093, OR 1.66; 95% CI (0.78-3.53).

Table 12: Allele distribution of TCF7L2 gene at rs12255372 between cases and control:



DISCUSSION

In this study, we have evaluated the association of TCF7L2 gene polymorphism with Type 2 Diabetes among 44 cases and 44 controls in Chennai suburban population. We have also attempted to evaluate the effect of gene polymorphism on GLP-1 and Insulin levels in the postprandial sample of age and gender matched study population.

The transcription factor TCF7L2 plays a key role in Wnt signaling pathway .TCF7L2 gene spans nearly 215.9Kbp containing 17 exons situated in high mobility group box. Wnt signaling is essential for glucose and lipid metabolism, functioning and proliferation of beta-cells of pancreas and GLP-1, the incretin hormone production[113]. Wnt activity is important particularly, for GLP-1 secretion in intestinal endocrine L-cells[113]. Any dysfunction in this pathway may lead to reduced GLP-1 secretion which may have effect on insulin secretion. Thus, GLP-1 plays a critical role in blood glucose homeostasis through insulin. The increased risk of T2DM conferred by variants in TCF7L2 involves the enteroinsular axis , impaired GLP-1 secretion and enhanced hepatic glucose production. The expression of TCF7L2 gene variants may dysregulate transcription of proglucagon gene that impairs GLP-1 secretion. This altered enteroinsular axis affects second phase of insulin secretion significantly. Reduced insulin secretion along with insulin resistance enhances hepatic glucose production leading to chronic hyperglycemic status.

It has been postulated that TCF7L2 gene polymorphism may confer susceptibility to T2DM by indirectly altering GLP-1 levels[114]. It was foreseen that in TCF7L2 risk allele carriers, the Wnt signaling is potentially increased in adipose tissue leading to

undifferentiation, impared growth and development of pre-adipocytes , that may influence BMI and waist circumference with its attendant sequelae[114,115].Thus, TCF7L2 might affect adipogenesis in peripheral tissues leading to insulin resistance[117].

The results in our study are similar to earlier findings that were done in Indian[25,113,116,])as well as non-Indian population[108,118-130].It shows a strong association of TCF7L2 variant with T2DM. This study not only restated the association of TCF7L2 with T2DM but also its effect on gene product ,GLP-1 and Insulin . GLP-1 levels are reduced in risk genotype carriers.

Thus our study reiterates TCF7L2 as the most important T2DM susceptible gene in our study population which has been universally replicated. Of the two SNPs, rs7903146 is significantly associated with T2DM which is consistent with other studies in Indian population[25,113,116,131-133] as well as in migrant Indian population[134]. Although the frequency of T allele in our study population was similar to frequency in Chandak et al[116], there is a lower frequency of T allele in diabetic subjects of our population than that reported in western Indian population[116].

In our study the TCF7L2 variant at rs12255372 did not show association with T2DM and the results are consistent with studies done by Uma Jyothi et al, in south Indian population where this variant at rs12255372 shows reduced risk compared to other sites[113]. T allele did not show any difference between cases and controls in our study which is in accordance with study by Mohan et al, where T allele frequency at rs12255372 is less in cases as compared to its frequency in controls[25,113].

Various studies that predicted the strong association of TCF7L2 gene polymorphism at rs7903146 and type 2 diabetes are.

- Grant et al., 2006; demonstrated a strong association in Icelandic population .Compared with non-carriers, heterozygous and homozygous carriers of the at-risk alleles (38% and 7% of the population, respectively) have relative risks of 1.45 and 2.41. In Icelandic cohort P = 2.1 × 10-9 .This was replicated in a Danish cohort (P = 4.8 × 10-3) and in a US cohort (P = 3.3 × 10-9[108].
- Groves et al., 2006; replicated the association in UK population.
- Damcott et al .,2006; replicated the association in Amish population. They established a strong association of TCF7L2 gene polymorphism at rs7901695 and rs7903146 (*P* = 0.008–0.01; OR 1.53–1.57) and marginal association with rs11196205 and rs12255372 (*P* = 0.07 and *P* = 0.04, respectively) with Type 2 Diabetes in Amish population.[65]
- Scott et al.,2006; replicated in French population. Both the T-allele of rs7903146 and the T-allele of rs12255372 significantly increase Type 2 Diabetes risk with an allelic odds ratio (OR) of 1.69 (95% CI 1.55–1.83) (*P* = 6.0 × 10−35) and 1.60 (1.47–1.74) (*P* = 7.6 × 10−28), respectively[136].
- In Chinese population association of TCF7L2 gene polymorphism at rs7903146 ,T allele was associated with an increased risk for T2DM under a dominant model, a co-dominant model and an allele contrast model, with an OR of 1.54 (1.32, 1.79), an OR of 1.53 (1.31, 1.79) and an OR of 1.52 (1.31, 1.76), respectively. The rs290487 C allele showed no significant overall association with T2DM, yielding ORs of 1.08 (0.88, 1.32) under a dominant model, with strong evidence of heterogeneity[135]
- In India , a study was conducted in Hyderabad , the diabetic capital of India .It is a case-control study of the three SNPs of TCF7L2, rs7903146, rs12255372 and

rs11196205, genotyped on Sequenom Massarray platform, in a sample of 758 patients and 621 controls. The greatest risk of developing the disease was conferred by rs7903146, implicating susceptibility for diabetes ($p<0.01$)[113].

- In Chennai , the study on TCF7L2 gene polymorphism show, T allele of the rs12255372(G/T) and rs7903146(C/T) confer more susceptibility to type 2 diabetes mellitus in Asian Indian[25].

GLP-1 the incretin hormone which is the gene product of TCF7L2 shows significantly reduced levels in 2-hour postprandial sample in type 2 diabetic cases than controls ,further strengthens our finding that the CT genotype of TCF7L2 variant at rs7903146, is a risk genotype for type 2 diabetes mellitus. This finding is against the reports done in German study population[137].

In our study, 2-hour postprandial insulin levels did not show difference between cases and controls This might be due to relatively small size of the study population and other interfering mechanisms from insulin anabolism to catabolism that alters insulin level which is in accordance with the study done in Spanish population where the 2-hours postprandial insulin level did not show any difference but proinsulin to insulin ratio was higher in risk genotype carrying T2DM cases than controls[138]. Though the exact mechanism of TCF7L2 gene is not known, it was postulated that TCF7L2 gene polymorphic variants have effect on proinsulin processing in the beta cells of pancreas that may predispose to type 2 diabetes[138].

CT genotype T2DM cases had high 2-hour plasma glucose levels and this further strengthens our study findings that T allele at rs7903146 is a risk allele for type 2 diabetes.

This finding is similar to studies by Chandak et al[116] and Lehman et al[139] who also reported increased 2-hour plasma glucose level in subjects with rs7903146 variants.

The postprandial lipid profile did not show any difference between diabetic group and control group. The fasting lipid profile was also not significantly different in similar studies among Indian population [113]. Recent studies indicate that for the cardiovascular risk assessment in T2DM, postprandial lipid profile could identify the risk factors better than fasting lipid profile[140]. In the cardiovascular risk assessment in Type 2 DM subjects, it is important to routinely estimate the postprandial lipid profile, in addition to the fasting lipid parameters. Atherosclerosis is a postprandial phenomenon with respect to lipids, as we are in the postprandial phase for most of the day, with an additional adverse effect of the meal induced hyperglycaemia. Also the total cholesterol, TGL and LDL levels were elevated in postprandial state as compared to fasting state in T2DM also HDL levels were lower in postprandial than in fasting. Rectifying the abnormal postprandial lipid parameters early in the course of diabetes, we can prevent the hazardous complications which are associated with Type 2 DM, the most common one being atherosclerotic coronary artery disease.

Despite the promising evidence for the association of TCF7L2 gene polymorphism at rs7903146 with Type 2 Diabetes, the precise mechanism behind TCF7L2 SNPs, which are identified in the intronic regions, is still unclear. It is a must to elude the mechanism of intronic SNPs that affect the expression of TCF7L2.Earlier studies showed the expression of TCF7L2 in gut than pancreatic islets. Henceforth, more research is focused on functional significance of this gene and more evidences are needed to study if TCF7L2 variants leads to alternative splicing and gene expression or change in protein structure.

It is essential to assess the role of different genes in the cause for Type 2 Diabetes in Chennai, other regions of Tamilnadu and India which has high prevalence of T2DM. This gauges enormous ethnic, geographic, genetic and cultural heterogeneity in the risk genetic profile of the Indian population. In conclusion, our study reaffirms the association of rs7903146 polymorphism of the TCF7L2 gene with T2DM in the Chennai suburban population in southern India.

SUMMARY

In Type 2 Diabetes, although there is considerable debate as to the relative contributions of beta-cell dysfunction and reduced insulin sensitivity to its pathogenesis, both these factors play important roles. But the mechanisms controlling the interplay of these two defects are unclear. A number of factors and mechanisms have been suggested till date.

Production and secretion of insulin from the β-cells of the pancreas is very crucial in maintaining normal glucose level. This is achieved by tight regulation of insulin synthesis and exocytosis from the β-cells in response to changes in blood glucose levels and incretin hormones specifically by GLP-1 and the regulation is at transcriptional, post-transcriptional and post-translational levels. The incretin GLP-1 is an insulinotropic hormone that potentially affect blood insulin level in the postprandial state. Through Wnt signaling, TCF7L2 the main transcription factor plays an important role in glucose induced - insulin gene transcription and pancreatic β-cell function.

TCF7L2 is the only transcription factor with the strongest effect on type 2 diabetes identified to date. But the molecular mechanism as to how variation in the gene increases the risk remains elusive. The phenotype changes associated with the risk genotype suggest that TCF7L2 polymorphism affect several vital functions leading to impaired pancreatic islet function and reduced islet mass , through GLP-1 and also directly that manifests as Type 2 Diabetes mellitus.

The gene expression of TCF7L2 variants , was found to exhibit an upregulation of mRNA levels and down regulation of protein level in specific cells[141].TCF7L2 ,a Wnt signaling – associated transcription factor is expressed in several tissues, including Gut and pancreas. Since 2006, the strong association of single nucleotide polymorphisms in the TCF7L2 gene at rs7903146 and rs 12255372 that are located between exons 3 and 4 and exons 4 and 5, respectively with type 2 diabetes has been confirmed in numerous studies[108,142,145] .

The presence of risk alleles in TCF7L2 gene are associated with specific T2DM phenotypes characterized by

- early impairment of beta- cell function,
- a reduction in GLP-1 induced potentiation of insulin secretion
- fasting and postprandial hyperglycemia in prediabetics [144,145].

This study, done on 44 type 2 diabetic cases and 44 healthy controls, who were age and gender matched ,to see the association of TCF7L2 gene polymorphism and ty 2 DM, revealed:

1. For rs7903146 (C/T) polymorphism,

- There is an increased frequency of risk allele " T" in cases (30.7%) than controls (2.3%) with a $p<0.001$.
- The GLP-1 level is reduced in both cases and controls with risk genotype CT when compared to subjects with CC genotype.
- The Insulin level show a significant difference between cases and controls with CT genotype .

- Presence of risk T allele coincides with hyperglycemic status in both cases and controls.

2. At rs 12255372 (G/T) polymorphism,

There is no significant increase in risk "T" allele distribution among cases and controls p=0.093.

Our study revealed a strong association of TCF7L2 gene polymorphism at rs7903146 with Type 2 Diabetes.

CONCLUSION

From the above discussion with regard to the results of the study, the conclusions arrived at are:

- TCF7L2 gene polymorphism at rs7903146 is strongly associated with type 2 diabetic patients in Chennai suburban population,
- The risk allele "T" and genotype " CT" is more commonly associated with diabetic cases than healthy controls.
- GLP-1 level is reduced in subjects with CT genotype than those with CC genotype in the study population , showing the effect of TCF7L2 gene polymorphism on GLP-1 production.
- CT genotype is associated with Postprandial hyperglycemic status than CC genotype in study subjects which emphasizes the role of TCF7L2 in maintaining blood glucose level.
- TCF7L2 gene polymorphism at rs 12255372 does not show significant association with type 2 diabetic patients in Chennai suburban population . This differs from previous studies in Chennai, mandating large study groups in this study area to assess the frequency of distribution for TCF7L2 gene polymorphism- rs12255372 .

The increased risk of T2DM conferred by variants in TCF7L2 involves the enteroinsular axis , impaired GLP-1 secretion and enhanced hepatic glucose production.

.

LIMITATIONS

This study with the population of 88 subjects had 44 diabetics as cases and 44 age and gender matched apparently normal subjects as controls, was done to look for an association of TCF7L2 gene polymorphism in Type 2 Diabetes.

Though the study revealed a strong association of TCF7L2 gene polymorphism at rs7903146, there was no significant association at rs12255372. This could be still due to lack of adequate samples where in all genotypic variants may not be included. To confirm the lack of association the study needs to be replicated in a large sample so as to include all the genetic variants. Only this can aid in documenting the lack of association at rs12255372 being true.

To look for the effect of polymorphism in blood glucose homeostasis, it would have been better if 1-hour post meal insulin and GLP-1 levels were measured, as the effect of incretin is to increase the second phase of insulin secretion.

Additionally, Proinsulin to insulin ratio gives an insight into the processing of insulin from its precursor for which the effect of GLP-1 is essential. This needs to be done in 2-hour post meal sample.

To document the effect of TCF7L2 gene polymorphism and its role in blood glucose homeostasis, assessment of insulin should be done in fasting,1-hour post meal and 2-hour post meal samples which emphasis the facts of basal insulin secretion ,the effect of incretin in increasing the second phase of insulin secretion and processing of insulin from its precursor recpectively.

Lipid profile diagnostically is always done in a fasting sample to assess the cardiovascular risk in T2DM patients. Recent studies have revealed that a postprandial sample is better for risk assessment as atherosclerosis is a postprandial phenomenon and we are in the postprandial state for most of the day. But the lack of reference values for postprandial lipid levels hinders the interpratation of lipid levels in T2DM patients for risk assessment. Hence it would have been better if the lipid profile was assayed in both the fasting and postprandial states.

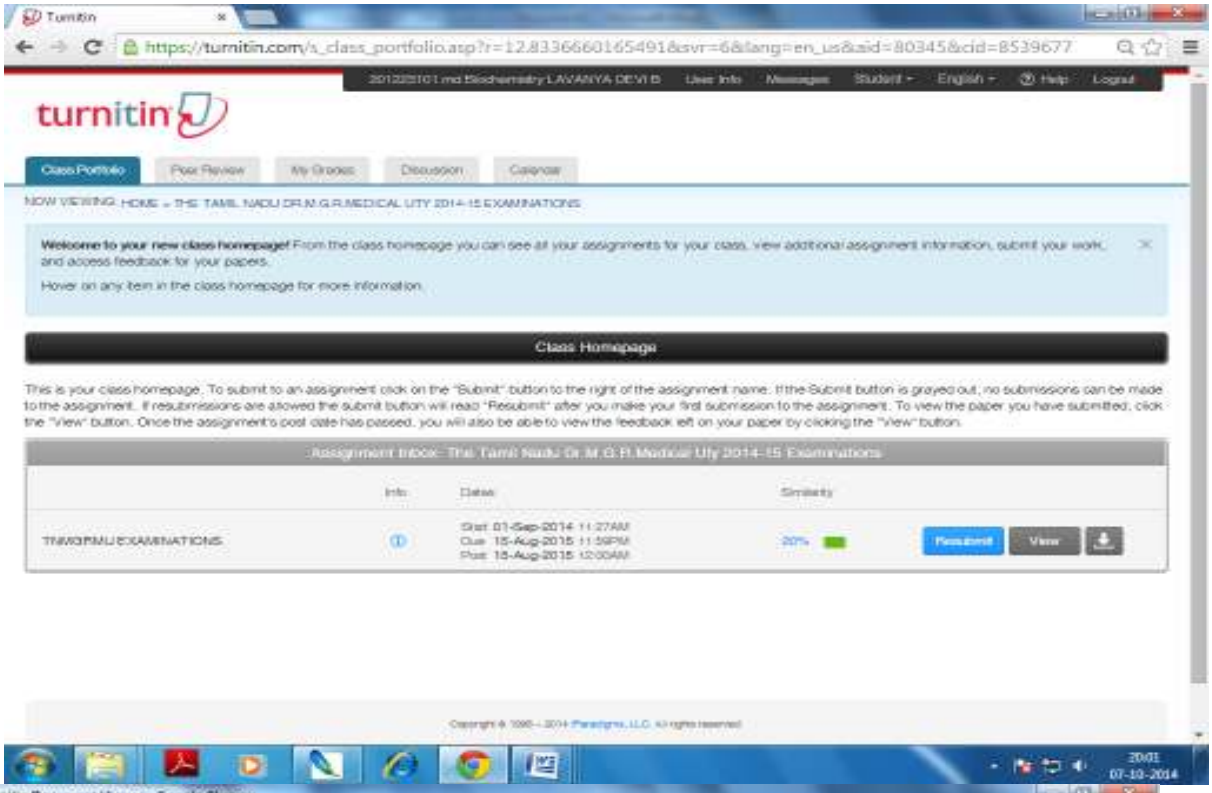
SCOPE FOR FURTHER STUDY

Further studies are eagerly needed, to translate the GWAS (Genome Wide Associatio Study) data to clinical interpretations. The regulation of TCF7L2 variants and the functional consequences at the protein level are still poorly understood. A clear picture of the molecular mechanism will be necessary to understand how an intronic variation in TCF7L2 can influence islet function.

TCF7L2 polymorphism leads to various splice variants which are differently regulated on the post-transcriptional level and would encode less active isoforms showing different action on beta-cell function and survival.

- Quantitative assessment of TCF7L2 and its correlation with GLP-1 levels would better help to identify the association of TCF7L2 polymorphism and its influence on protein levels.
- Due to TCF7L2 polymorphism ,if GLP-1 level is altered needs further studies.
- Action of TCF7L2 gene in adipose tissue and its role in lipid metabolism needs further research.
- Measurement of GLP-1 and Insulin in 1 hour postprandial sample documents the effect of incretin on the second phase of insulin secretion.
- Proinsulin to insulin ratio measurement aids in understanding the effect of GLP-1 on insulin processing.

TCF7L2 gene involved in regulating glucose homeostasis needs further exploration . This may aid in genetic diagnosis and early prevention of Type 2 Diabetes. TCF7L2 shows the largest effective size with an odds ratio (OR) of 1.37, followed by KCNQ1 with second largest effective size with OR of 1.29 (Yasuda et al., 2008; Unoki et al., 2008). However the molecular mechanism by which they exert their biological functions is still not known. So, further association studies for TCF7L2and KCNQ1 and other variants are needed to assess their role in other populations, especially populations with a high prevalence of Type 2 Diabetes such as in India.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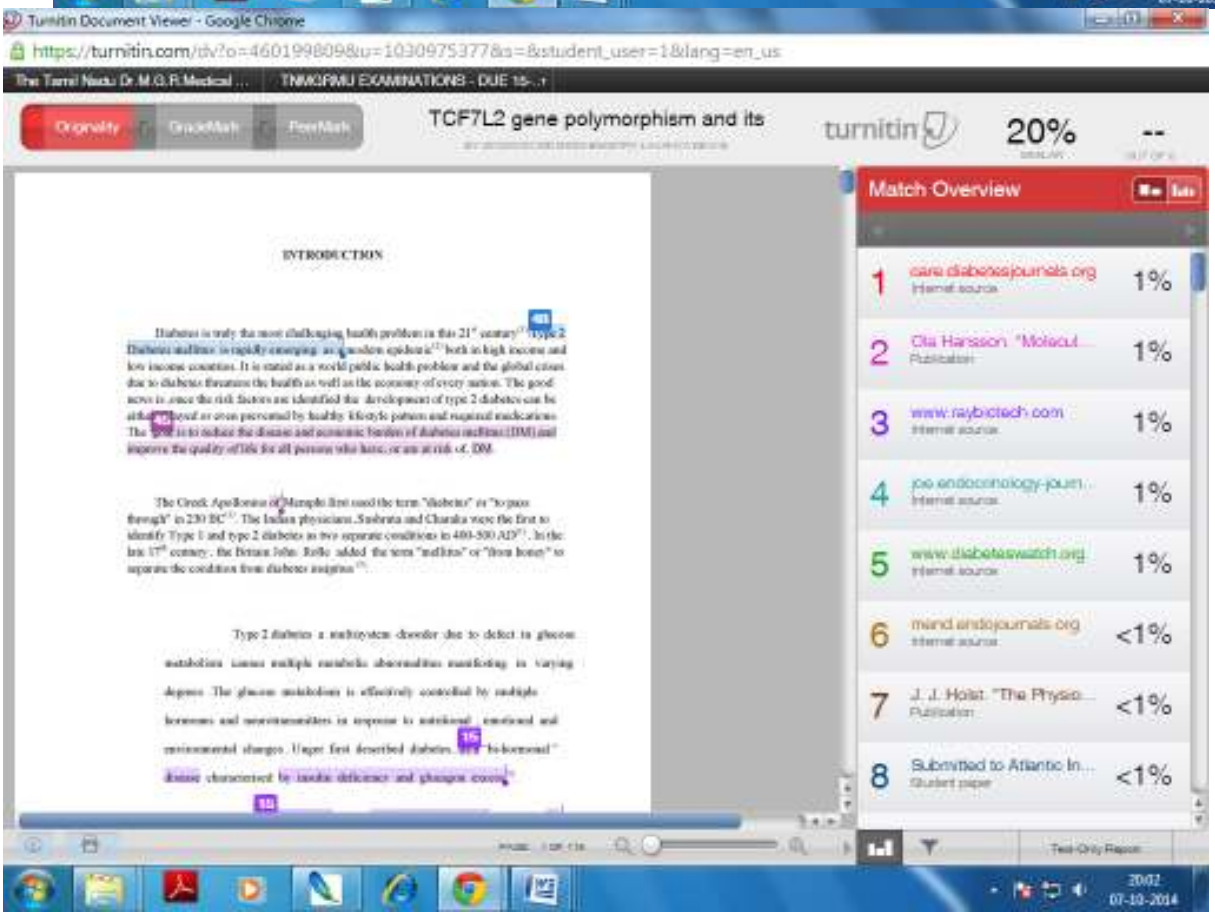
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INTRODUCTION

INSTITUTIONAL ETHICAL COMMITTEE
GOVT.KILPAUK MEDICAL COLLEGE, CHENNAI-10

Ref.No.3393/ME-1/Ethics/2013 Dt:27.09.2013

CERTIFICATE OF APPROVAL

The Institutional Ethical Committee of Govt. Kilpauk Medical College, Chennai reviewed and discussed the application for approval "A Study on TCF7L2 polymorphismsrs12255372 (G/T) and rs7903146 (C/T) and their association with type 2 diabetes mellitus" – For Project Work Submitted by Dr.B.Lavanya Devi, MD (Bio-Chem), Student, KMC, Chennai.

The Proposal is APPROVED.

The Institutional Ethical Committee expects to be informed about the progress of the study any Adverse Drug Reaction Occurring in the Course of the study any change in the protocol and patient information /informed consent and asks to be provided a copy of the final report.

5 NOV 2013

25/11/13

CHAIRMAN,
Ethical Committee
Govt.Kilpauk Medical College,
Chennai

நோயாளி ஒப்புதல் படிவம்

ஆராய்ச்சியின் விவரம் : **TCF7L2** மரபணு பாளிமார்பிசம் நீரிழிவு நோய் - வகை 2
ஆராய்ச்சி மையம் - அரசு கீழ்பாக்கம் மருத்துவக் கல்லூரி மருத்துவமனை

நோயாளியின் பெயர் : நோயாளியின் வயது : ஆ/பெ
பதிவு எண் : முகவரி :

1. மேற்குறிப்பிட்டுள்ள ஆராய்ச்சியின் நோக்கத்தையும் பயனையும் முழுவதுமாக புரிந்து கொண்டேன். மேலும் எனது அனைத்து சந்தேகங்களையும் கேட்டு அதற்கான விளக்கங்களையும் தெளிவுபடுத்திக்கொண்டேன்.
2. மேலும் இந்த ஆராய்ச்சிக்கு எனது சொந்த விருப்பத்தின் பேரில் பங்கேற்கிறேன் என்றும், மேலும் எந்த நேரத்திலும் எவ்வித முன்னறிவிப்புமின்றி இந்த ஆராய்ச்சியிலிருந்து விலக முழுமையான உரிமை உள்ளதையும், இதற்கு எவ்வித சட்ட பிணைப்பும் இல்லை என்பதையும் அறிவேன்.
3. ஆராய்ச்சியாளரே, ஆராய்ச்சி உதவியாளரோ, ஆராய்ச்சி உபயத்தாரோ, ஆராய்ச்சி பேராசிரியரோ, ஒழுங்குநெறி செயற்குழு உறுப்பினர்களோ எப்போது வேண்டுமானாலும் எனது அனுமதியின்றி எனது உள்நோயாளி பதிவுகளை இந்த ஆராய்ச்சிக்காகவோ அல்லது எதிர்கால பிற ஆராய்ச்சிகளுக்காகவோ பயன்படுத்திக் கொள்ளலாம் என்றும் மேலும் இந்த நிபந்தனை நான் இவ்வாராய்ச்சியிலிருந்து விலகினாலும் தரும் என்றும் ஒப்புக் கொள்கிறேன், ஆயினும் எனது அடையாளம் சம்பந்தப்பட்ட எந்த பதிவுகளும் (சட்டபூர்வமான தேவைகள் தவிர) வெளியிடப்படமாட்டாது என்ற உறுதிமொழியின் பெயரில் இந்த ஆராய்ச்சியிலிருந்து கிடைக்கப்பெறும் முடிவுகளைவெளியிட மறுப்பு தெரிவிக்கமாட்டேன் என்று உறுதியளிக்கின்றேன்,
4. இந்த ஆராய்ச்சிக்கு நான் முழு மனதுடன் சம்மதிக்கின்றேன் என்றும், மேலும் ஆராய்சிசிக் குழுவினர் எனக்க அளிக்கும் அறிவுரைகளை தவறாது பின்பற்றுவேன் என்றும் உறுதியளிக்கின்றேன்.
5. இந்த ஆராய்சிசிக்குத் தேவைப்படும் அனைத்து மருத்துவப் பரிசோதனைகளுக்கும் ஒத்துழைப்பு தருவேன் என்று உறுதியளிக்கின்றேன்.
6. இந்த ஆராய்ச்சியில் சக்கரை நோய்கான பரிசோதனைகளுக்கும், மேலும் மரபணு சோதனையும் மேற்கொள்ளப்படுகிறது என்பதை ஆராய்ச்சியாளர் மூலம் அறிந்து கொண்டேன். மரபணு சோதனைக்கும் எனது முழு ஒப்புதலை தருகிறேன்.
7. இந்த ஆராய்சிக்கு யாருடைய வற்புறுத்தலுமின்றி எனது சொந்த விருப்பத்தின் பேரிலும் சுய அறிவுடனும் முழுமனதுடனும் சம்மதிக்கின்றேன் என்று இதன் மூலம் ஒப்புக் கொள்கிறேன்.

ஆராய்ச்சியாளரின் கையொப்பம் :

இடம் :
தேதி :

நோயாளியின் கையொப்பம் / பெருவிரல் கைரேகை :

இடம் :
தேதி :

PATIENT CONSENT FORM

Study Detail	:	**TCF7L2 Gene Polymorphism in Type 2 Diabetes**
Study Centre	:	**Govt.Kilpauk Medical College and Hospital**
Patient's Name	:	**Age/ Gender: M/F**
Identification Number	:	**Address :**

I confirm that I have understood the purpose and procedure of the above study. I had the opportunity to ask questions and all my questions and doubts have been answered to my complete satisfaction.

I understand that my participation in the study is voluntary and that I am free to withdraw at any time without giving , without my rights being affected.

I understand that the sponsor of the clinical study, others working on the sponsor' s behalf, the ethical committee and the regulatory authorities will not need my permission to look at my health records, both in respect of the current study and my further research that may be conducted in relation to it, even if I withdraw from the study I agree to this access. However I understand that my identity would not be revealed in any information released to third partiesor published, unless as required under the law. I agree not to restirct the use of any data or results that arise from this study.

I understand that the study involves, investigations regarding not just insulin assay, but also involves gene analysis of TCF7L2 GENE

I hereby consent to participate in this study including for gene analysis.

I hereby give permission to undergo complete clinical examination and diagnostic tests including haematological, radiological tests.

Signature of the Investigaor :

Name of the investigator:

Place :

Date :

Signature / Thumb impression

Place :

Date :

PROFORMA

Name : Age: M/F

OP/IP no : Address :

Occupation:

Presenting Complaints:

Past H/O:

DM / HT/ Hypercholestrolemia/ Hypothyroid/ IHD/ Endocrine Disorder/ Renal Disease/ Hepatic Disease.

Treatment H/O: [exclusions-on insulin,GLP1 analogs]

Drug- single/ combination

Personal H/O: smoking /alcohol/tobacco chewing

Family H/o:

O/E:

Built - obese/thin/moderate **BMI:**

Height- Weight- Waist-

Pedal edema /Anemia / Lymphadenopathy-

Vitals:

BP: Pulse Rate:

Systemic examination:

CVS: RS:

CNS: Abdomen:

Diagnosis:

Investigations:

Blood sugar –

Sr.Urea :

Sr. Creatinine:

T.Cholestrol:

HDLc: (Direct)

Non-HDLc : (calculated)

HbA1C:

Insulin:

GLP-1:

TCF7L2 Gene Polymorphism: rs 7903146- CC / CT / TT

rs 12255372- GG /GT / TT

ABBREVIATION

ADA- American Diabetes Association

WHO-World Health Organization

GWAS-Genome Wide Association Studies

IDF-International Diabetes Federation

NGSP- National Glycohemoglobin Standardisation Program

DCCT- Diabetic Control and Complications Trial

GLP- Glucose - dependent Insulinotropic Polypeptide

GLP-1- Glucagon - Like Peptide-1

NGT- Normal Glucose Tolerance

PGDP-Pro-Glucagon Derived Peptide

GRPP- Glicentin – Related Pancreatic Polypeptide

MPGF- Major Proglucagon Fragment

PC- Prohormone convertase

GEFII - Guanine nucleotide Exchange Factor II

CIE - Ca^{2+} dependant Insulin Exocytosis

cAMP- cyclic Adenosine Mono Phosphate

PKA- Protein Kinase A

PG - Pro-Glucagon fragment

IP-1 - Intervening Peptide-1

IP-2 - Intervening Peptide-2

gcg - glucagon gene

LRP - Lipoprotein Receptor-related Protein

Ryk - Receptor tyrosine kinase

Dsh/Dvl - Dishevelled

HMG - High Mobility Group box

TLE - Transducin-like Enhancer

TCF7L2 -Transcription factor 7 – like 2

CtBP-1 - C-terminal binding protein-1

SNP – Single Nucleotide Polymorphism

BIBLIOGRAPHY

1. Shaw JE1, Sicree RA, Zimmet PZ (2010) Global estimates of the prevalence of diabetes for 2010 and 2030. Diabetes Res Clin Pract 87: 4-14.

2. Wild S1, Roglic G, Green A, Sicree R, King H (2004) Global prevalence of diabetes: estimates for the year 2000 and projections for 2030. Diabetes Care 27: 1047-1053.

3. Stephen L. Aronoff, Kathy Berkowitz, Barb Shreiner, and Laura Want. Glucose Metabolism and Regulation: Beyond Insulin and Glucagon Diabetes Spectrum. Volume 17, Number 3, 2004.

4. Ralph A. DeFronzo. From the Triumvirate to the Ominous Octet: A New Paradigm for the Treatment of Type 2 Diabetes Mellitus. DOI: 10.2337/db09-9028, From the Diabetes Division, University of Texas Health Science Center, San Antonio, Texas, Diabetes, vol. 58, April 2009.

5. Abdulfatai B. Olokoba,1, Olusegun A. Obateru,2 and Lateefat B. Olokoba3, Type 2 Diabetes Mellitus: A Review of Current Trends, Oman Med J. Jul 2012; 27(4): 269–273.

6. McCarthy MI. Genomics, type 2 diabetes and obesity. N Engl J Med 2010; 363:2339–2350.

7. Horikawa Y1, Oda N, Cox NJ et al 2000, Genetic variation in the gene encoding calpain-10 is associated with type 2 diabetes mellitus. Nat Genet. 2000 Oct;26(2):163-75.

8. MauraAgostini,ErikSchoenmakers,CatherineMitchel et al,2006, on-DNA binding, dominant-negative, human PPARγ mutations cause lipodystrophic insulin resistance. Cell Metab. Oct 2006; 4(4): 303–311.

9. Sunita Singh, THE GENETICS OF TYPE 2 DIABETES MELLITUS : A REVIEW. Banaras Hindu University, Varanasi ISSN : 0447-9483

10. Bodhini D., Radha V., Dhar M., Narayani N., Mohan V. The rs12255372(G/T) and rs7903146(C/T) polymorphisms of the TCF7L2 gene are associated with type 2 diabetes mellitus in Asian Indians. Metabolism. 2007;56:1174–1178.

11. Chandak G.R., Janipalli C.S., Bhaskar S., Kulkarni S.R., Mohankrishna P., Hattersley A.T., Frayling T.M., Yajnik C.S. Common variants in the TCF7L2 gene are strongly associated with type 2 diabetes mellitus in the Indian population. Diabetologia.2007;50:63–67.

12. Drucker DJ. The biology of incretin hormones. Cell Metab.2006;3(3):153-65.

13. World Health Organisation .Definition, Diagnosis, and classification of Diabetes Mellitus.WHO/NCD/NCS/99 .2 ed .Geneva, World Health Organisation, 1999.

14. Lei Chen, Dianna J. Magliano & Paul Z. Zimmet . The worldwide epidemiology of type 2 diabetes mellitus—present and future perspectives. Nature Reviews Endocrinology 8, 228-236 ,April 2012.

15. Institute of medicine. Appendix D. Ambulatory care sensitive conditions and referral sensitive surgeries. Access to health care in America . Washington , DC : National academy press, 1993: 219-222 .

16. Wild S, Roglic G, Green A, Sicree R, King H. Global prevalence of diabetes: estimates for the year 2000 and projections for 2030. Diabetes Care. 2004; 27(5):1047–53.

17. International Diabetes Federation (2013) IDF Diabetes Atlas (6thedn). Brussels, Belgium: International Diabetes Federation.

18. "IDF Diabetes Atlas." International Diabetes Federation. November 2012. Web. March 2013. http://www.idf.org/diabetesatlas.

19. Reshma S Patil[1], Jayashree S Gothankar[1]. Prevalence of Type-2 Diabetes Mellitus and Associated Risk Factors in an Urban Slum of Pune City, India. Natl J Med Res. 2013; 3(4): 346-349.

20. Mohan V1, Sandeep S, Deepa R, Shah B, Varghese C. Epidemiology of type 2 diabetes: Indian scenario. Indian J Med Res. 2007 Mar;125(3):217-30.

21. American Diabetes Association-2012 : Diagnosis and Classification of Diabetes Mellitus. DOI:10.2337/dc12-S064.

22. Standards of Medical Care in Diabetes – 2014. American Diabetes Association . Diabetes Care Volume 37, Supplement 1, January 2014 . DOI: 10.2337/dc14-S014.

23. Viswanathan Mohan, Janarthanan Vijay Venkatraman, and Rajendra Pradeepa. Epidemiology of Cardiovascular Disease in Type 2 Diabetes: The Indian Scenario Journal of Diabetes Science and Technology. Volume 4, Issue 1, January 2010.

24. Naveed Sattar* and Jason MR Gill. Type 2 diabetes as a disease of ectopic fat? Sattar and Gill BMC Medicine 2014, 12:123.

25. Mohan V, Deepa R, Rani SS, Premalatha G, Chennai UrbanPopulation Study (CUPS No. 5). Prevalence of coronary artery disease and its relationship to lipids

in a selected population in South India: the Chennai Urban Population Study. J Am Coll Cardiol. 2001;38(3):682–7.

26. NP Steyn, J Mann, PH Bennett, N Temple, P Zimmet, J Tuomilehto, J Lindstro M and A Louheranta . Diet, nutrition and the prevention of type 2 diabetes Public Health Nutrition: 7(1A),147–165.

27. Jenifer Lovejoy and Mario DiGirolamo . Habitual dietary intake and insulin sensitivity in lean and obese adults. Am J Clin Nutr 1992; 55 : 1174-9.

28. Genetics expression Staal FJ, Clevers H. tcf/f lef transcription factors during T cell development: unique and overlapping functions. Hematol J. 2000; 1 (1):3-6.

29. Owerbach, D., Bell, G. I., Rutter, W. J., Brown, J. A., andShows, T. B.: The insulin gene is located on the short arm of chromosome II in humans. Diabetes 1981; 30:267-70.

30. M. Alan permutt, John chirgwin, Peter rotwein, and Steve giddings, Diabetes care 1984; 7:386-94.

31. Pfeffer SR, Rothman JE. Biosynthetic protein transport and sorting by the endoplasmic reticulum and golgi. Ann Rev Biochem 1987;56:829-852.

32. Orci L. The insulin factory :a tour of the plant surroundings and a visit to the assembly line. Diabetologia 1985;28:528-546 .

33. Rhodes CJ, Halban PA.newly synthesized proinsulin/insulin and stored insulin are released from pancreatic B-cells via a regulated, rather than a constitutive pathway.J Cell Biol 1987;105:145-153.

34. .Proks P,Eliasson L, Ammala C, et al.Ca2+ and GTP – dependant exocytosis in in mouse pancreatic β cells involves both common and distinct steps. J Physiol 1996;496:255.

35. Bratanova – Tochkoya TK, Cheng H,Daniel S,et al.Triggering and augmentation mechanisms, granule pools and biphasic insulin secretion. Diabetes 2002;51(suppl):83)(Olofsson CS, Gopel SO,Barg S,et al.fast insulin secretion reflects exocytosis of docked granules in mouse pancreatic B-cells. Pflugers Arch 2002;444:43

36. O'Connor MD, Landahl H, Grodsky GM. Comparison of storage- and signal-limited models of pancreatic insulin secretion. Am J Physiol. 1980 May;238(5):R378-89.

37. Frances m. Ashcroft, donna e. Harrison & stephen j. H. Ashcroft. Glucose induces closure of single potassium channels in isolated rat pancreatic β-cells .Nature 312, 446 - 448.

38. Jean-Claude Henquin. Triggering and Amplifying Pathways of Regulation of Insulin Secretion by Glucose.Diabetes 2000; 49:1751.

39. Aizawa T, Komatsu M, Asanuma N, et al. Glucose action 'beyond ionic events' ij the pancreatic β cell. Trends Pharmacol Sci 1998;19: 496.

40. Unger, R. H., and A. M. Eisentraut. 1969. Entero-insular axis. Arch. Intern. Med. 123:261-266.

41. Creutzfelt, W. 1979. The incretin concept today. Diabetologia. 16:75-85.

42. John I Stagner and Ellis Samols. The Vascular Order of Islet Cellular Perfusion in the Human Pancreas.Diabetes 1992;41:93-97).

43. George G. Holz and Joel F. Habener. Signal transduction crosstalk in the endocrine system: pancreatic β-cells and the glucose competence concept. Trends Biochem Sci. Oct 1992; 17(10): 388–393.

44. Heller RS1, Kieffer TJ, Habener JF .Insulinotropic glucagon-like peptide I receptor expression in glucagon-producing alpha-cells of the rat endocrine pancreas. Diabetes. 1997 May;46(5):785-91.

45. Miholic J1, Orskov C, Holst JJ, Kotzerke J, Meyer HJ. .Emptying of the gastric substitute, glucagon-like peptide-1 (GLP-1), and reactive hypoglycemia after total gastrectomy. Dig Dis Sci. 1991 Oct;36(10):1361-70 .

46. Bernard Thorens .Cell Biology Expression cloning of the pancreatic β cell receptor for the gluco-incretin hormone glucagon-like peptide 1 (insulin secretion/non-insulin-dependent diabetes mellitus/entero-insular axis/G proteins/cAMP). Proc. Nati. Acad. Sci. USA Vol. 89, pp. 8641-8645, September 1992.

47. Svetlana Mojsov, Gerhard Heinrich, Ira B. Wilson, Mariella Ravazzolall, Lelio Orcin, and Joel F. Habener .Preproglucagon Gene Expression in Pancreasa nd Intestine Diversifies at the Level of Posttranslational Processing. The American Society of Biological Chemists. , Inc Vol 261, No. 25, Issue of September 5.

48. Tianru Jin. Review . Mechanisms underlying proglucagon gene expression. Journal of Endocrinology (2008) 198, 17–28.

49. James W.White and Grady F.Saunders .Nucleic Acids Research , Structure of the human glucagon gene, Volume 14 ,Number 12, 1986 .

50. **Daniel J Drucker.** Biologic actions and therapeutic potential of the proglucagon-derived peptide.www.nature.com/clinicalpractice . 19August 2005.

51. Yi F, Sun J, Lim GE, Fantus IG, Brubaker PL, Jin T 2008 Crosstalk between the insulin and Wnt signaling pathways: evidence from intestinal endocrine L cells. Endocrinology 149:2341–2351.

52. Patzelt C and Schiltz E (1984) Conversion of proglucagon in pancreatic alpha cells: the major end products are glucagon and a single peptide, the major proglucagon fragment, that contains two glucagon-like sequences. Proc Natl Acad Sci USA 81:5007–5011 .

53. Tianru Jin and Ling Liu. The Wnt Signaling Pathway Effector TCF7L2 and Type 2 Diabetes Mellitus. Molecular Endocrinology 22(11):2383–2392.

54. JENS JUUL HOLST. The Physiology of Glucagon-like Peptide 1. Department of Medical Physiology, The Panum Institute, University of Copenhagen, Copenhagen, Denmark. Physiol Rev 87: 1409–1439, 2007.

55. Orskov C . Glucagon-like peptide-1, a new hormone of the entero-insular axis .Diabetologia. 1992 Aug ; 35 (8) : 701-11.

56. Frank Reimann, Molecular mechanisms underlying nutrient detection by incretin-secreting cells. 2009 Elsevier International Dairy Journal 20 (2010) 236–242

57. Linda M. Morgan, Peter R. Flatt and Vincent Marks.. Nutrient Regulation Of The Enteroinsular Axis And Insulin Secretion. Nutrition Research Reviews (1988),1,79-97.

58. Wook Kim and Josephine M. Egan. The Role of Incretins in Glucose Homeostasis and DiabetesTreatment. Pharmacol Rev. 2008 December ; 60(4): 470–512.

59. Non epi in starvation] Zauner, C., Schneeweiss, B., Kranz, A., Madl, C., Ratheiser, K., Kramer, L., & Lenz, K. (2000). Resting energy expenditure in short-term starvation is increased as a result of an increase in serum norepinephrine. The American journal of clinical nutrition, 71(6), 1511-1515.

60. Orci L, Malaisse-Lagae F, Amherdt M,Ravazzola M, Weisswange A, Dobbs RD, Perrelet A, Unger R: Cell contacts in human islets of Langerhans. J Clin Endocrinol Metab 41:841–844, 1975.

61. Gerich J, Davis J, Lorenzi M, Rizza R,Bohannon N, Karam J, Lewis S, Kaplan R, Schultz T, Cryer P: Hormonal mechanisms of recovery from hypoglycemia in man. Am J Physiol 236:E380–E385, 1979.

62. Alisa Peet MD , Michael A. Lieberman, Allan Marks MD Lieberman, Marks's Basic Medical Biochemistry,Fourth Edition .

63. Michael W King. Lipolysis and the Oxidation of Fatty Acids1996–2014 the medical biochemistry page.org, May 1, 2014.

64. Pehling G, Tessari P, Gerich JE, Haymond MW, Service FJ, Rizza RA: Abnormal meal carbohydrate disposition in insulin-dependent diabetes: relative contributions of endogenous glucose production and initial splanchnic uptake and effect of intensive insulin therapy. J Clin Invest 74:985–991, 1984.

65. Coleen M. Damcott , Toni I. Pollin , Laurie J. Reinhart , Sandra H. Ott , Haiqing Shen, Kristi D. Silver, Braxton D. Mitchell1and Alan R. Shuldiner. Polymorphisms in the transcription factor 7-like 2 (TCF7L2) gene are associated with type 2 diabetes in the Amish: replication and evidence for a role in both insulin secretion and insulin resistance. Diabetes . 2006 Sep; 55(9):2654-9.

66. A. Holdcroft. Hormones and the Guts. British Journal of Anesthesia 85 (1) 58-68(2000).

67. Muhammad A. Abdul-Ghani. Type 2 Diabetes and the Evolving Paradigm in Glucose Regulation. Am J Manag Care. 2013;19(3 suppl):S43-S50.

68. Ola Hansson, Yuedan Zhou, Erik Renström,Peter Osmark. Molecular Function of TCF7L2: Consequences of TCF7L2 Splicing for Molecular Function and Risk for Type 2 Diabetes. Lund University Publications. Current Diabetes Reports 2010 Okt.

69. Grzeskowiak R1, Amin J, Oetjen E, Knepel W. Insulin responsiveness of the glucagon gene conferred by interactions between proximal promoter and more distal enhancer-like elements involving the paired-domain transcription factor Pax6. J Biol Chem. 2000 Sep 29;275(39):30037-45.

70. Ahlgren U1, Pfaff SL, Jessell TM, Edlund T, Edlund H. Independent requirement for ISL1 in formation of pancreatic mesenchyme and islet cells. Nature. 1997 Jan 16;385(6613):257-60.

71. Philippe J1, Morel C, Prezioso VR.Glucagon gene expression is negatively regulated by hepatocyte nuclear factor 3 beta. Mol Cell Biol. 1994 May;14(5):3514-23.

72. Turton MD et al. (1996) A role for glucagon-like peptide-1 in the central regulation of feeding. Nature 379: 69–72.
73. 36 Flint A et al. (1998) Glucagon-like peptide 1 promotes satiety and suppresses energy intake in humans. J Clin Invest 101: 515–520.

74. Zhu X et al. (2002) Disruption of PC1/3 expression in mice causes dwarfism and multiple neuroendocrine peptide processing defects. Proc Natl Acad Sci USA 99: 10293–10298.

75. Drucker DJ et al. (1996) Induction of intestinal epithelial proliferation by glucagon-like peptide 2. Proc Natl Acad Sci USA 93: 7911–7916.

76. Wynne K et al. (2005) Subcutaneous oxyntomodulin reduces body weight in overweight and obese subjects: a double-blind, randomized, controlled trial. Diabetes 54: 2390–2395.

77. Orskov C, Holst JJ, Poulsen SS, Kirkegaard P. Pancreatic and intestinal processing of proglucagon in man. Diabetologia 30: 874–881, 1987.

78. Orci L, Bordi C, Unger RH, Perrelet A. Glucagon- and glicentin producing cells. In: Glucagon, edited by Lefebvre PJ. Berlin: Springer-Verlag, 1983, p. 57–79.

79. Yi F, Sun J, Lim GE, Fantus IG, Brubaker PL, Jin T 2008 Crosstalk between the insulin and Wnt signaling pathways: evidence from intestinal endocrine L cells.Endocrinology 149:2341–2351.

80. Meier JJ, Nauck MA, Kranz D, Holst JJ, Deacon CF, GaecklerD, Schmidt WE, Gallwitz B. Secretion, degradation, elimination of glucagon-like peptide 1 and gastric inhibitory polypeptide in patients with chronic renal insufficiency and healthy control subjects.Diabetes 53: 654–662, 2004.

81. Vilsboll T, Agerso H, Krarup T, Holst JJ. Similar elimination rates of glucagon-like peptide-1 in obese type 2 diabetic patients and healthy subjects. J Clin Endocrinol Metab 88: 220–224, 2003.

82. Oben J, Morgan L, Fletcher J, Marks V. Effect of the enteropancreatic hormones, gastric inhibitory polypeptide and glucagonlike polypeptide-1 (7–36) amide, on fatty acid synthesis in explants of rat adipose tissue. J Endocrinol 130: 267–272, 1991.

83. Vilsboll T, Krarup T, Sonne J, Madsbad S, Volund A, Juul AG, Holst JJ. Incretin secretion in relation to meal size and body weight in healthy subjects and people with type 1 and type 2 diabetes mellitus. J Clin Endocrinol Metab 88: 2706–2713, 2003.

84. Miholic J, Orskov C, Holst JJ, Kotzerke J, Meyer HJ. Emptying of the gastric substitute, glucagon-like peptide-1 (GLP-1), reactive hypoglycemia after total gastrectomy. Dig Dis Sci 36: 1361–1370, 1991.

85. Elliott RM, Morgan LM, Tredger JA, Deacon S, Wright J, Marks V. Glucagon-like peptide-1 (7–36) amide and glucose-dependent insulinotropic polypeptide secretion in response to nutrient ingestion in man: acute post-prandial and 24-h secretion patterns. J Endocrinol 138: 159–166, 1993.

86. Nauck MA, Niedereichholz U, Ettler R, Holst JJ, Orskov C, Ritzel R, Schmiegel WH. Glucagon-like peptide 1 inhibition of gastric emptying outweighs its insulinotropic effects in healthy humans. Am J Physiol Endocrinol Metab 273: E981–E988, 1997.

87. Yu-tingA. Chiang, Wilfred Ip and Tianru Jin. The role of the Wnt signaling pathway in incretin hormone production and function. Frontiers in Physiology.Review Article published:12July2012.

88. D'Alessio D, Vahl T, Prigeon R 2004 Effects of glucagon-like peptide 1 on the hepatic glucose metabolism. Horm Metab Res 36:837–841.

89. Rania Abu-Hamdah, Atoosa Rabiee, Graydon S. Meneilly, Richard P. Shannon, Dana K. Andersen, and Dariush Elahi. The Extrapancreatic Effects of Glucagon-Like Peptide-1 and Related Peptides. J Clin Endocrinol Metab. Jun 2009; 94(6): 1843–1852.

90. Ahrén B 2004 GLP-1 and extra-islet effects. Horm Metab Res 36:842–845 .

91. Laurie L. Baggio and Daniel J. Drucker. Biology of Incretins: GLP-1 and GIP. Gastroenterology 2007;132:2131–2157.

92. Delmeire D, Flamez D, Hinke SA, Cali JJ, Pipeleers D, and Schuit F. Type VIII adenylyl cyclase in rat _ cells: coincidence signal detector/generator for glucose and GLP-1. Diabetologia 46:1383–1393, 2003.

93. Hisatomi M, Hidaka H, and Niki I. $Ca2^{+}$/calmodulin and cyclic3',5' adenosine monophosphate control movement of secretory granules through protein phosphorylation/dephosphorylation in the pancreatic β-cell. Endocrinology 137: 4644–4649, 1996.

94. Susumu Seino and Tadao Shibasaki. PKA-Dependent and PKA-Independent Pathways for cAMP-Regulated Exocytosis. Physiol Rev 85: 1303–1342, 2005.

95. Mayo KE, Miller LJ, Bataille D, Dalle S, Goke B, Thorens B,Drucker DJ. International Union of Pharmacology. XXXV. The glucagon receptor family. Pharmacol Rev 2003;55:167–194.

96. Frank Reimann. Molecular mechanisms underlying nutrient detection by incretin-secreting cells. International dairy journal ,april 2010;20(4):236-242.

97. Tianru Jin , Mechanisms underlying proglucagon gene expression. J Endocrinol July 1, 2008 198 17-28.

98. Martin J. Seidensticker, Jurgen Behrens. Biochemical interactions in the wnt pathway. Biochimica et Biophysica Acta 1495 (2000) 168-182.

99. Tata Purushothama Rao and Michael Kuhl.An Updated Overview on Wnt Signaling Pathways: A Prelude for More. Circ Res. 2010;106:1798-1806.

100. Raymond Habas and Igor B Dawid.Dishevelled and Wnt signaling: is the nucleus the final frontier? .Journal of Biology 2005, 4:2.

101. Chien AJ, Conrad WH, Moon RT: A Wnt survival guide: from flies to human disease. J Invest Dermatol 2009, 129:1614–1627.

102. Britta Wallmen, Monika Schrempp and Andreas Hecht. Intrinsic properties of Tcf1 and Tcf4 splice variants determine cell-type-specific Wnt/b-catenin target gene expression. Nucleic Acids Research, 2012, 1–15.

103. Srinivas D. Narasipura,a Lisa J. Henderson,a Sidney W. Fu,b Liang Chen,b Fatah Kashanchi,c and Lena Al-Harthia. Role of β-Catenin and TCF/LEF Family Members in Transcriptional Activity of HIV in Astrocytes. Journal of Virology p. 1911–1921.

104. Luan Shu1, Aleksey V. Matveyenko, Julie Kerr-Conte, Jae-Hyoung Cho,Christopher H.S. McIntosh and Kathrin Maedler. Decreased TCF7L2 protein levels in type 2 diabetes mellitus correlate with downregulation of GIP- and GLP-1 receptors and impaired beta-cell function. Human Molecular Genetics, 2009, Vol. 18, No. 13 2388–2399.

105. Tianru Jin and Ling Liu. The Wnt Signaling Pathway Effector TCF7L2 and Type 2 Diabetes Mellitus. Molecular Endocrinology 22(11):2383–2392.

106. Shitashige M, Hirohashi S, Yamada T: Wnt signaling inside the nucleus. Cancer Sci 2008, 99:631–637.

107. Ford CE, Ekstrom EJ, Howlin J, et al.: The WNT-5a derived peptide, Foxy-5, possesses dual properties that impair progression of ERalpha negative breast cancer. Cell Cycle 2009, 8:1838–1842.

108. Struan F A Grant1, Gudmar Thorleifsson1, Inga Reynisdottir1 et al., 2006.Variant of transcription factor 7-like 2 (TCF7L2) gene confers risk of type 2 diabetes. Nature Genetics 38, 320 - 323 (2006) .

109. HapMap [http://www.hapmap.org] webcite

110. Slattery ML, Folsom AR, Wolff R, Herrick J, Caan BJ, Potter JD (April 2008). "Transcription factor 7-like 2 polymorphism and colon cancer".Cancer Epidemiol. Biomarkers Prev. 17 (4): 978–82

111. Osmark P, Hansson O, Jonsson A, et al.: Unique splicing pattern of the TCF7L2 gene in human pancreatic islets. Diabetologia 2009, 52:850–854.

112. David E. Reich1, Michele Cargill1,2, Stacey Bolk1, James Ireland1, Pardis C. Sabeti3, Daniel J. Richter1, Thomas Lavery1, Rose Kouyoumjian1, Shelli F. Farhadian1, Ryk Ward3 & Eric S. Lander1,4 Linkage disequilibrium in the human genome. Nature 411, 199-204 (10 May 2001) | doi:10.1038/35075590; Received 11 December 2000; Accepted 13 March 2001.

113. Kommoju Uma Jyothi, Maruda Jayaraj, Kadarkarai Samy Subburaj, Kotla Jaya Prasad,Irgam Kumuda, Velaga Lakshmi, Battini Mohan Reddy. Association of TCF7L2 Gene Polymorphisms with T2D in the Population of Hyderabad, India. April 2013 | Volume 8 | Issue 4 .

114. Smith U. (2007) TCF7L2 and type 2 diabetes-weWNT to know. Diabetologia 50: 5–7.

115. Gupta V, Khadgawat R, Saraswathy KN, Sachdeva MP, Kalla AK. (2008). Emergence of TCF7L2 as a most promising gene in predisposition of Diabetes Type II. Int J Hum Genet,8: 199–215.

116. Chandak GR, Janipalli CS, Bhaskar S, Kulkarni SR, Mohankrishna P, et al.(2007) Common variants in the TCF7L2 gene are strongly associated with type 2 diabetes mellitus in the Indian population. Diabetologia 50: 63–67.

117. Damcott CM, Pollin TI, Reinhard LJ, Ott SH, Shen H, et al. (2006) Polymorphisms in the transcription factor 7-like 2 (TCF7L2) gene are associated with type 2 diabetes in the Amish: replication and evidence for a role in both insulin secretion and insulin resistance. Diabetes 55: 2654–2659.

118. Scott LJ, Bonnycastle C, Willer CJ, Sprau AG, Jackson AU, et al. (2006) Association of transcription factor 7-like 2 (TCF7L2) variants with type 2diabetes in a Finnish sample. Diabetes 55: 2649–2653.

119. Damcott CM, Pollin TI, Reinhard LJ, Ott SH, Shen H, et al. (2006) Polymorphisms in the transcription factor 7-like 2 (TCF7L2) gene are associated with type 2 diabetes in the Amish: replication and evidence for a role in both insulin secretion and insulin resistance. Diabetes 55: 2654–2659.

120. Zhang C, Qi L, Hunter DJ, Meigs JB, Manson JE, et al. (2006) Variant of transcription factor 7-like 2 (TCF7L2) gene and the risk of type 2 diabetes in large cohorts of U.S. women and men. Diabetes 55: 2645–2648.

121. Moczulski D, Gawlik B, August R, Strojek K, Grzeszczak W.(2007) TCF7L2 gene is associated with type 2 diabetes in Polish population. Exp Clinic Diabetologia 7: 109–111.

122. Lyssenko V, Lupi R, Marchetti P, Del Guerra S, Orho-Melander M, et al.(2007) Mechanisms by which common variants in the TCF7L2 gene increaserisk of type 2 diabetes. J Clin Invest 117: 2155–2163.

123. Ng MC, Tam CHT, Lam VK, So WY, Ma RC, et al. (2007) Replication and identification of novel variants at TCF7L2 associated with type 2 diabetes in Hong Kong Chinese. J Clin Endocrinol Metab 92: 3733–3737.

124. 124. Marquezine GF, Pereira AC, Sousa AG, Mill JG, Hueb WA, et al.(2008).TCF7L2 variant genotypes and type 2 diabetes risk in Brazil: significant association, but not a significant tool for risk stratification in the general population. BMC Med Genet 9: 106.

125. Hayashi T, Iwamoto Y, Kaku K, Hirose H, Maeda S.(2007) Replication study for the association of TCF7L2 with susceptibility to type 2 diabetes in a Japanese population. Diabetologia 50: 980–984.

126. Horikoshi M, Hara K, Ito C, Nagai R, Froguel P, et al. (2007) A genetic variation of the transcription factor 7-like 2 gene is associated with risk of type 2 diabetes in the Japanese population. Diabetologia 50: 747–751.

127. Kunika K, Tanahashi T, Numata S, Ueno S, Ohmori T, et al. (2008) Common coding variant in the TCF7L2 gene and study of the association with type 2 diabetes in Japanese subjects. J Hum Genet 53: 972–982.

128. Mayans S, Lackovic K, Lindgren P, Ruikka K, Agren A, et al. (2007) TCF7L2 polymorphisms are associated with type 2 diabetes in northern Sweden. Eur J Hum Genet 15: 342–346.

129. De Silva NM, Steele A, Shields B, Knight B, Parnell K, et al. (2007) The transcription factor 7-like 2 (TCF7L2) gene is associated with Type 2 diabetes in UK community-based cases, but the risk allele frequency is reduced compared with UK cases selected for genetic studies. Diabet Med 24: 1067–1072.

130. Van Vliet-Ostaptchouk JV, Shiri-Sverdlov R, Zhernakova A, Strengman E, vanHaeften TW et al. (2007) Association of variants of transcription factor 7-like

2(TCF7L2) with susceptibility to type 2 diabetes in the Dutch Breda cohort. Diabetologia 50: 59–62.

131. Chauhan G, Spurgeon CJ, Tabassum R, Bhaskar S, Kulkarni SR, et al. (2010) Impact of common variants of PPARG, KCNJ11, TCF7L2, SLC30A8, HHEX, CDKN2A, IGF2BP2,and CDKAL1 on the risk of type 2 diabetes in 5,164 Indians. Diabetes 59: 2068–2074.

132. Sanghera DK, Nath SK, Ortega L, Gambarelli M, Kim-Howard X, et al. (2008) TCF7L2 polymorphisms are associated with type 2 diabetes in Khatri Sikhs from North India: genetic variation affects lipid levels. Ann Hum Genet 72: 499– 509.

133. Sanghera DK, Ortega L, Han S, Singh J, Ralhan SK, et al. (2008) Impact of nine common type 2 diabetes risk polymorphisms in Asian Indian Sikhs: PPARG2 Pro12Ala),IGF2BP2, TCF7L2 and FTO variants confer a significant risk. BMC Med Genet 9: 59.

134. Rees SD, Bellary S, Britten AC, O'Hare JP, Kumar S, et al. (2008) Common variants of the TCF7L2 gene are associated with increased risk of type 2 diabetes mellitus in a UK-resident South Asian population. BMC Med Genet 9: 8.

135. Zhang BC1, Li WM, Zhu MY, Xu YW.Association of TCF7L2 gene polymorphisms with type 2 diabetes mellitus in Han Chinese population: a meta-analysis. Gene. 2013 Jan 1;512(1):76-81. Epub 2012 Sep 23.

136. Stéphane Cauchi, David Meyre, Christian Dina et al., 2006. Transcription Factor *TCF7L2*Genetic Study in the French Population Expression in Human β-Cells and Adipose Tissue and Strong Association With Type 2 Diabetes. Diabetes. 2006 Oct;55(10):2903-8.

137. S. A. Schäfer, O. Tschritter, F. Machicao, et al., 2007. Impaired glucagon-like peptide-1-induced insulin secretion in carriers of transcription factor 7-like 2 (TCF7L2)gene polymorphisms. Diabetologia (2007) 50:2443–2450.

138. J. L. González-Sánchez & M. T. Martínez-Larrad & C. Zabena & M. Pérez-Barba &M. Serrano-Ríos Association of variants of the TCF7L2 gene with increases in the risk of type 2 diabetes and the proinsulin:insulin ratio in the Spanish population Diabetologia (2008) 51:1993–1997.

139. Lehman DM, Hunt KJ, Leach RJ, Hamlington J, Arya R, Abboud HE, et al. Haplotypes of transcription factor 7–like 2 (TCF7L2) gene and its upstream region are associated with type 2 diabetes and age of onset in Mexican Americans. Diabetes 2007;56:389-93.

140. Lokhande Suryabhan L, Iyer Chandrashekhar M, Shinde Ratnendra R, Nandedkar Prerna DComparative Study on the Fasting and the Postprandial Dyslipidaemia in Type 2 Diabetes Mellitus Journal of Clinical and Diagnostic Research. 2013 April, Vol-7(4): 627-630.

141. Le Bacquer O1, Shu L, Marchand M, Neve B, Paroni F, Kerr Conte J, Pattou F, Froguel P, Maedler K. TCF7L2 splice variants have distinct effects on beta-cell turnover and function. Hum Mol Genet. 2011 May 15;20(10):1906-15.

142. Lyssenko V.The transcription factor 7-like 2 gene and increased risk of type 2 diabetes: an update. Curr. Opin. Clin. Nutr. Metab. Care 2008;11:385-392.

143. Perry J.R., Frayling T.M.New gene variants alter type 2 diabetes risk predominantly through reduced beta-cell function. Curr. Opin. Clin. Nutr. Metab. Care 2008;11:371-377.

144. Nauck M.A., Meier J.J.The enteroinsular axis may mediate the diabetogenic effects of *TCF7L2* polymorphisms. Diabetologia 2007;50:2413-2416.

145. Lyssenko V., Lupi R., Marchetti P., DelGuerra S., Orho,Melander M., AlmgrenP., S jogren M., Ling C., Eriksson K.F., Lethagen A.L., et al. Mechanisms by which common variants in the *TCF7L2* gene increase risk of type 2 diabetes. J. Clin Invest. 2007;117:2155-2163.

146. Leonid Poretsky, (2009). Principles of diabetes mellitus (2nd ed.). New York: Springer. p. 3. ISBN 978-0-387-09840-1 .

147. Ludmila Alves Sanches DutraI; Patrícia Godoy Garcia CostaI; Lara Franciele Ribeiro VelascoI; Angélica Amorim AmatoII; Gustavo Barcelos BarraIAllele-specific PCR assay to genotype SNP rs7903146 in TCF7L2 gene for rapid screening of diabetes susceptibility. Arq Bras Endocrinol Metab vol.52 no.8 São Paulo Nov. 2008.

Master table showing characteristics of type 2 diabetes mellitus patients (1 to 44) and control (45 to 88):

	Age	Gender	Height	Weight	Waist	INSULIN	GLP-1	Glucose	Urea	Cr	T,Cho	HDL	Non-HDL	HbA1c	rs12255372	rs7903146
1	48	F	151	65	101	48.3	-1.812	253	29.5	0.56	190	44.4	146	8.6	GT	CC
2	48	F	157	77	107	41.2	-2.891	209	29.8	0.74	172	54.8	117	6.8	GG	CT
3	63	F	164	95	135	40.1	-1.07	90.8	22.8	0.93	171	55.1	116	7.6	GG	CT
4	42	M	157	57	97	421	-2.138	127	29.1	1.04	162	33.1	129	5.5	GG	CT
5	63	F	146	55	107	35.8	-3.153	380	31.1	0.7	168	40.5	127	9.8	GT	CT
6	66	F	152	65	110	782	-3.851	281	16.8	0.84	155	36.6	118	8.6	GG	CT
7	62	F	155	64	101	78.4	-3.436	221	28.5	0.91	222	43.8	178	6.9	GT	CT
8	53	M	154	55	97	103	-3.201	233	20.5	1.11	185	55.9	129	7.7	GG	CC
9	70	F	145	57	110	407	-1.914	246	29.5	1.03	200	50.7	150	7.8	GT	CT
10	40	F	147	67	107	45	-3.594	128	20.2	0.76	160	40.4	120	6.1	GG	CT
11	48	M	147	52	98	34.9	-1.052	333	28.1	0.87	172	57.1	114	11.1	GT	CC
12	37	M	146	70	118	47	-1.475	330	17.7	0.64	166	35.2	131	12.2	GG	CC
13	49	M	170	47	91	414	-1.719	302	20.8	0.82	147	42.7	104	9.1	GT	CC
14	55	M	157	55	95	925	-2.696	199	22.9	0.78	162	44.2	118	7.5	GG	CC

15	59	M	155	95	139	238	-1.749	115	27.8	0.66	138	30	108	6.1	GG	CC
16	54	F	150	68	122	25.7	-2.151	229	21.7	0.8	165	40.8	124	8.6	GT	CT
17	45	M	167	72	104	36.1	-0.605	350	27.4	1.04	201	50.1	151	11	GG	CC
18	54	M	165	79	121	35.7	0.232	91.8	26.6	1.1	171	24.2	147	5.3	GG	CC
19	67	M	165	55	92	40.7	0.892	205	31.9	0.93	151	37.7	113	8.8	GT	CT
20	45	F	145	45	97	13.5	0.644	405	25.8	0.9	180	59	121	10.7	GT	CT
21	44	F	156	43	90	687	0.385	284	19.2	0.77	151	45	106	8.5	GG	CT
22	48	M	145	54	104	28.5	0.387	303	23	0.84	138	40.4	97.7	8.5	GG	CC
23	46	F	148	42	92	194	1.89	108	20.3	0.96	146	45.9	99.8	9.9	GG	CT
24	40	F	148	57	102	24.5	-0.357	209	24.7	0.81	170	43.3	126	7.5	GG	CT
25	51	F	147	55	110	49.5	0.815	345	30.5	0.96	213	54.5	158	11.1	GT	CT
26	38	F	150	65	105	10.5	0.89	197	24.5	0.84	144	33.7	111	7.2	GT	CT
27	52	F	160	60	105	713	0.978	281	14.2	0.73	165	50.4	115	9.1	GG	CT
28	45	F	157	82	127	40.3	0.507	152	23.3	0.73	155	43.6	112	6.4	GT	CT
29	47	F	147	47	96	1077	0.828	259	24.2	0.63	221	52.1	169	10.1	GT	CT
30	50	F	148	51	90	646	0.241	155	21.8	0.75	223	42.1	181	6.6	GT	CT
31	37	M	160	70	104	383	0.542	188	18.8	0.73	157	26.7	130	7.9	GT	CC

32	52	M	151	57	102	891	0.134	272	14.8	0.77	153	50.1	103	9	GT	CC
33	64	M	160	63	99	91.3	8.958	299	28.1	1.16	172	31.7	140	7.7	GT	CC
34	52	F	150	55	87	80.4	0.468	156	23	0.73	239	45	194	6.5	GT	CT
35	40	F	147	50	92	704	0.776	393	20.8	0.84	202	61.5	140	11.5	GG	CT
36	46	M	160	62	94	22.6	0.579	235	23.3	0.94	182	41.9	141	6.5	GG	CT
37	43	M	167	55	95	1123	0.926	409	27.9	1.14	148	33.5	115	12	GG	CT
38	47	M	165	74	106	602	0.382	165	26.9	1.09	172	41.7	131	6.3	TT	CC
39	52	F	143	47	96	542	0.453	228	18.7	0.82	214	32.3	182	10.7	GG	CT
40	43	M	160	63	100	142	4.144	231	20.4	0.98	148	41.3	107	6.7	GG	CC
41	42	M	150	50	96	7.17	1.525	350	21.7	0.67	143	44.2	99.2	10.6	GG	CC
42	57	F	153	70	111	40	4.712	295	28.9	0.91	237	45.8	192	7.9	GG	CC
43	43	M	170	65	101	188	0.448	274	20.3	1.06	190	51.5	139	8.7	GT	CT
44	42	F	155	45	96	45.2	1.673	255	30.6	0.9	172	52.3	120	7.6	GG	CT
45	47	M	155	65	90	348	0.673	129	34	1.15	198	47	151	5.7	GG	CC
46	53	M	162	65	89	378	0.387	164	25	0.8	177	51.7	125	5.2	GT	CC
47	57	M	150	68	101	595	-0.126	108	26.4	0.91	182	45.6	137	5.3	GG	CC
48	47	M	145	52	87	261	0.752	112	24	0.92	156	41	115	5.4	GG	CC

49	44	F	145	65	109	9.52	0.926	84.9	18.4	0.82	159	42.5	117	5.1	GG	CT
50	41	F	140	63	105	45.9	0.944	138	16.1	0.76	162	46.7	115	5.6	GT	CT
51	47	M	167	70	91	489	0.786	116	28	0.93	167	43.8	123	5	GG	CC
52	47	F	141	55	81	48	0.278	130	28.2	0.82	159	53.9	105	5.2	GT	CC
53	49	F	151	45	80	32.2	-0.631	97.3	23	0.85	151	48.4	103	5.3	GG	CC
54	52	F	139	40	82	347	-0.036	98.6	25.6	0.96	162	48.1	114	5.5	GG	CC
55	40	F	162	65	91	984	0.096	72.9	18.2	0.68	210	47.8	162	4.7	GG	CC
56	47	F	163	60	86	33.3	0.458	91.3	17.9	0.82	171	41.6	129	5.5	GG	CC
57	51	F	161	60	88	18.9	3.761	72.9	20.7	0.93	170	39.3	131	5.3	GG	CC
58	42	M	150	54	85	24.2	8.929	101	24.6	1.01	155	39.3	115	5	GG	CC
59	42	F	150	59	85	46.4	2.636	96.6	23.9	0.9	167	54	113	5.6	GG	CC
60	36	F	162	60	90	7.09	8.726	81.9	21.2	0.89	192	54.6	137	5	GG	CC
61	37	F	158	61	90	188	2.55	103	21.8	0.84	166	58.8	108	4.9	GG	CC
62	45	F	142	65	95	40.4	0.83	69.5	14.7	0.89	156	46.1	110	5.6	GG	CC
63	46	F	162	55	75	14.7	0.918	124	22.3	0.81	155	50	105	5.6	GT	CC
64	48	F	145	69	105	7.73	4.463	92.7	23.8	1.17	179	44.1	134	5	GG	CC
65	39	F	145	55	85	23.3	4.178	129	16.7	0.82	161	43.1	118	5.2	GG	CC

66	42	F	155	65	95	44.4	7.359	96.1	24.3	0.94	160	37.9	122	5.3	GG	CC
67	43	M	160	69	99	220	0.687	130	29.1	1.01	168	49.8	118	5.2	GG	CC
68	65	M	170	79	100	872	0.862	168	24.3	1.08	144	36.8	107	5.2	GG	CC
69	54	F	145	60	90	484	4.826	130	31.6	0.85	134	55.6	78.7	5.6	GG	CC
70	61	M	160	70	102	49.7	9.923	82.6	27.1	1.24	187	43.5	143	4.9	GG	CC
71	69	M	170	85	95	712	3.513	90.4	21.7	1.18	181	41	140	5.1	GT	CC
72	44	M	150	65	99	59.3	7.575	119	24.5	1.43	178	44.1	134	5.2	TT	CC
73	49	F	154	67	95	23.6	9.868	98.7	30.7	0.88	174	39.9	135	4.2	TT	CC
74	44	F	140	55	104	309	1.969	133	26.1	1.04	225	54.6	170	5.4	GG	CC
75	43	M	155	65	95	20.2	6.962	98.3	23.4	1.14	160	43.9	116	5.1	GG	CC
76	54	M	145	60	90	48.9	4.434	107	35.5	0.93	137	40	96.5	4.8	GG	CC
77	65	M	160	70	100	15.5	1.316	89.5	39.7	1.15	171	37.1	134	5.4	GG	CC
78	53	F	155	65	95	27.8	5.215	82.5	29.9	1.02	189	57.1	132	5	GT	CC
79	55	M	158	69	102	10.7	7.698	87.1	34.4	0.82	169	31.9	137	4.1	GG	CC
80	67	M	157	60	90	9.04	8.735	113	39.7	0.79	157	52.1	105	5	GG	CC
81	46	F	169	90	101	36.6	2.051	143	35.5	1.1	151	30.5	120	5.2	GT	CC
82	60	M	160	66	99	43.5	0.669	92.5	25.5	1.19	145	26.4	118	5.1	GG	CC

83	61	M	140	68	105	572	0.885	133	24.5	1.06	176	53.1	123	5.7	GT	CC
84	50	F	160	65	95	937	3.071	143	19.8	1	184	49.7	134	5.5	GG	CC
85	40	M	169	85	105	294	8.643	98.5	27.6	1.19	170	37.1	133	4.7	GG	CC
86	40	F	156	68	105	510	5.852	105	30.2	0.84	190	45.8	145	5.7	GT	CC
87	59	M	165	70	100	9.58	7.397	99	24.8	1.02	139	33.7	105	5.7	GG	CC
88	51	F	145	69	105	41.3	6.859	128	19.7	0.9	228	36.9	191	5.8	GT	CC